IDENTIFICATION AND ANTIBIOGRAM PROFILING OF *ESCHERICHIA COLI* AND *SALMONELLA* SPP. ISOLATED FROM SUPPLIED WATER OF SELECTED CATTLE FARMS IN DHAKA

MD. MASHIUR RAHMAN

DEPARTMENT OF MICROBIOLOGY AND PARASITOLOGY

SHER-E-BANGLA AGRICULTURAL UNIVERSITY

DHAKA-1207

DECEMBER, 2021

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By

MD. MASHIUR RAHMAN
Registration No: 14-06057

A Thesis

Submitted to the Faculty of Animal Science and Veterinary Medicine

Sher-e-Bangla Agricultural University, Dhaka-1207,

In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE (MS) IN MICROBIOLOGY
DEPARTMENT OF MICROBIOLOGY AND PARASITOLOGY

Semester: July-December, 2021

Approved By

Dr. Muhammad Abdul Mannan
Supervisor
Assistant Professor
Department of Microbiology & Parasitology
Sher-e-Bangla Agricultural University, Dhaka

Dr. Mahfuzul Islam
Co-Supervisor
Chairman & Associate Professor
Department of Microbiology & Parasitology
Sher-e-Bangla Agricultural University, Dhaka

Dr. Mahfuzul Islam
Associate Professor
Chairman, Examination Committee
Department of Microbiology and Parasitology
Sher-e-Bangla Agricultural University, Dhaka-1207

DEPARTMENT OF MICROBIOLOGY AND PARASITOLOGY

Sher-e-Bangla Agricultural University

Sher-e-Bangla Nagar, Dhaka-1207

Memo No: SAU/MIPA

CERTIFICATE

*This is to certify that the thesis entitled "**IDENTIFICATION AND ANTIBIOGRAM PROFILING OF ESCHERICHIA COLI AND SALMONELLA SPP. ISOLATED FROM SUPPLIED WATER OF SELECTED CATTLE FARMS IN DHAKA**" submitted to the Department of Microbiology and Parasitology, Faculty of Animal Science & Veterinary Medicine, Sher-e-Bangla Agricultural University, Dhaka-1207, as partial fulfillment for the requirements of the degree of Master of Science (MS) in Microbiology, embodies the result of a piece of bonafide research work carried out by **MD. MASHIUR RAHMAN,** Registration No.: **14-06057,** Session: **JUL-DEC/2019** under my supervision and guidance. No part of this thesis has been submitted for any other degree or diploma.*

I further certify that any help or source of information, received during the course of this investigation has been duly acknowledged.

November 2022
Dhaka, Bangladesh

Dr. Muhammad Abdul Mannan
(Supervisor)
Assistant Professor
Department of Microbiology and Parasitology
Sher-e-Bangla Agricultural University
Dhaka-1207


DEDICATED
TO
MY BELOVED PARENTS

ACKNOWLEDGEMENT

At the beginning, the author bows the grace and mercy of the "Almighty Allah," the omnipotent and omniscient, who enabled him to complete this thesis. The author expresses his grateful acknowledgement and indebtedness to his beloved father Mofijul Islam Akhan and mother Nasima Begum for their ending prayer, sacrifice and dedicated efforts to educate him this level.

The author with a sense of respect expresses his heartfelt gratitude to his honorable supervisor and Assistant Professor ***Dr. Muhammad Abdul Mannan****, Department of Microbiology and Parasitology, Sher-e-Bangla Agricultural University, Dhaka-1207 for his continuous inspiration, valuable suggestions, constructive criticism, constant guidance and intensive supervision throughout the period of the study. Preparation of this thesis without his intense co-operation would not have been possible.*

Heartfelt gratitude and profound respect to his honorable Co-supervisor ***Dr. Mahfuzul Islam,*** *Chairman & Associate Professor****,*** *Department of Microbiology & Parasitology****,*** *Sher-e-Bangla Agricultural University****,*** *Dhaka-1207****,*** *for his keen interest, valuable advice, creative suggestions, co-operation and encouragement to bring this thesis up to its present standard.*

It is also a great pleasure for the author to express hearty appreciation and regard to the honorable teacher, ***DR. Md. Kamrul Hassan,*** *Associate Professor, Department of Microbiology and Parasitology, Sher-e-Bangla Agricultural University, Dhaka-1207, for his affectionate feelings and valuable suggestions during the research work.*

The author humbly desires to express his profound respect & sincere appreciation to respectable teacher, ***prof. Dr. Uday Kumar Mohanta,*** *Professor, Department of Microbiology and Parasitology, Sher-e-Bangla Agricultural University, Dhaka-1207 for his co-operation & inspiration to conduct the academic work.*

The author is grateful to ***DR. S. M. Abdullah,*** *Assistant professor, Department of Microbiology and Parasitology, Sher-e-Bangla Agricultural University, Dhaka-1207 for his motivation & academic support.*

The author expresses his grateful thanks to all staffs and employees of the Department of Microbiology and Parasitology, Sher-e-Bangla Agricultural University, Dhaka 1207, for their co-operation and encouragement to prepare this thesis.

The author expresses his deepest sense of gratitude, indebtedness and profound respect to his beloved roomate DR. Imran Hossain, Md. Rasel Kabir and his friends DR. Saifullah Mahmood, DR. Nehal Hasnain, DR. Tanjila Mousum, DR. Sharmin Rahman, DR. Nipu Shen, DR. Al Wasif Jhontu, and DR. Maniruzamman for their blessings, encouragement and moral support in all phases of this academic pursuit from beginning to the end.

The Author

ABSTRACT

The present study was carried out to isolate, identify and to know antibiogram profiling of *Escherichia coli* and *Salmonella* spp. from the supplied drinking water in different cattle farms in Dhaka city within the period of October, 2020 to April, 2021. A total of 100 fresh water samples were randomly collected from different cattle farms and transported to the Microbiology and Parasitology laboratory of Sher-e-Bangla Agricultural University for microbiological analysis. Primary culture was done in nutrient broth and nutrient agar. Pure culture was obtained from different selective media. Bacteria were identified by observing the growth properties in different media, staining properties and biochemical tests. The characteristics of *E. coli* colonies were red to bright pink colored in MacConkey agar and greenish red colored with faint metallic sheen in EMB (Eosin Methylene Blue) agar. The characteristics of *Salmonella* spp. colonies were black colored in SS agar and pink colored in MacConkey agar. In Gram's staining, *E. coli* revealed gram-negative, pink color, small rod-shaped appearance, arranged in single or paired short, and *Salmonella* spp. revealed gram negative, short rod shaped, singly arranged. The occurrence of *E. coli* was 46% and *Salmonella* spp. was 37%. Pure isolates were subjected to antibiogram by disc diffusion method against 5 different antibiotics including streptomycin (Str), ampicillin (Amp), ciprofloxacin (Cip), gentamycin (Gen) and tetracycline (Te). Highest number of *E. coli* isolates showed resistance to gentamycin (43%) followed by ampicillin (38%) and tetracycline (26%). In case of *Salmonella* spp., highest number of *E. coli* isolates showed resistance to gentamycin (31%) followed by ampicillin (31%) and tetracycline (27%). This study revealed that water samples collected from different cattle farms of Dhaka city were contaminated with multiple species of multidrug resistant bacteria which may pose risk for both animal and human health.

Keywords: *E. coli*, *Salmonella*, cattle farm water, occurrence, antibiogram

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LIST OF ABBREVIATIONS

SAU	Sher-e-Bangla Agricultural University
Spp.	Species
NA	Nutrient agar
SS	Salmonella-Shigella
EMB	Eosin Methylene Blue
MR	Methyl-Red
MAC	MacConkey Agar Media
VP	Voges Proskauer
Gm	Gram
Cfu/ml	Colony forming unit per milliliter
NB	Nutrient broth
%	Percentage
+ve	Positive
-ve	Negative
Gm+ve	Gram positive
Gm-ve	Gram negative
MHA	Muller Hinton Agar
µg	Micro gram

CHAPTER 1

INTRODUCTION

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INTRODUCTION

Water is an essential nutrient required for all animals to survive (Acharjee *et al*., 2013). It makes up roughly 60% to 70% of an adult animal's live weight and about 80% of the live weight of a newborn (Weber *et al*., 1994). Water has multiple roles as a consumption item, production item, environmental amenity and is the backbone of human development. While most resources have substitutes, water fulfils a number of functions where there is no substitute. In spite of such importance, this is the resource which has been taken for granted and mismanaged for last so many decades. Drinking water can be considered an essential nutrient for dairy cattle and high yielding cows may drink up to 120 liters a day (Karanis *et al*., 2002). This water may originate from different sources and should be safe, palatable and with a low risk of precipitation in the water system. Sources of water include surface water from streams and rivers, lagoons and ponds, pumped well water, collected rain water and tap water. Water can become contaminated by faeces from humans, rodents, and birds, by recycled waste water and by cattle faeces. Bacteria, viruses, and parasites are potential microbial contaminants. While the quality of water samples taken from the source is often assessed, the quality of water at the end points of the distribution system, where cattle actually drink the water is assessed less often, even though there may be large differences in quality between source and end points (Caterina *et al*., 2012). This is because the micro environment of water distribution systems is quite complex and still not fully understood. Thus, the quality of water on dairy farms could be considered a determinant of herd health and productivity and even of public health or food safety.

Livestock play a crucial role in the agricultural production systems of Bangladesh. For the subsistence farm economy, livestock is an essential component. Livestock are also important sources of farmers' cash income and in the national economy livestock bring a significant portion of foreign exchange earnings through the export of hides. The livestock sector generates 20% of full-time employment in Bangladesh (DLS, 2013). According to DLS, the cattle population of Bangladesh in 2021 was about 25.7 million. According to BARC, average annual per-capita meat consumption is low (1.96 kg/person), most of which comes from cattle (1.25kg).Now the number of cattle farms is about 12 lakhs (1.2 million), including small and large ones (The Daily Star, 2019). About 3000 cattle farm is located in and around Dhaka city (The Daily Star, 2019). Maximum farm is for both dairy and beef purpose. Reliable, high quality water supply is

essential to dairy farms. Water is used for animal consumption, milk cooling, cleaning and sanitizing equipment, cow cooling. Supply water of Dhaka WASA is being used by the farms. Various pathogens like bacteria, virus, and fungus are present in supply water (Maringo *et al.,* 1992). *Escherichia coli* and *Salmonellae* is the most common bacteria in water. *E. coli* and *Salmonella* spp. in contact with surface or groundwater can survive for days to months, with the duration influenced in part by such factors as temperature and exposure to UV radiation (Bitton *et al.,* 1983). Low temperature can prolong the survival of generic *E. coli* and *E. coli* O157:H7 (Wang and Doyle, 1998). Elevated generic *E. coli* levels were observed in a large freshwater source when solar radiation decreased and cloud cover increased (Whitman *et al.,* 2004). It would be beneficial to have a better understanding of the causes of increased levels of *E. coli,* especially pathogenic *E. coli* and *Salmonella* in water on or near produce farms in order to better manage the risk of produce contamination. In the cattle gastrointestinal tract, *E. coli* O157:H7 is more often found in the hindgut as compared to the animals' rumen and one of the main colonization sites is the distal part of the rectum (Naylor *et al.,* 2003). *Escherichia coli* that did not belong to the O157:H7 serotype appeared to be distributed throughout the intestinal tract and their largest numbers were found in the large intestine.

E. coli is a large and diverse group of bacteria. Some kinds of *E. coli* can cause diarrhea, while others cause urinary tract infections, respiratory illness and pneumonia and other illnesses. Three groups of *E. coli* bacteria have been associated with diarrhea in calves (Jagals *et al.,* 2004). The most common are enterotoxigenic *E. coli* (ETEC). Enteropathogenic and enterohemorrhagic types are also common but are non-pathogenic to cattle, including the verocytotoxic (VTEC) forms that cause severe illness in humans such as *E. coli* 0157.

There are many *Salmonella* species that are able to infect cattle. It can cause a wide range of clinical signs in cattle including diarrhea and possible dysentery, joint infections, chronic pneumonia, abortion and sudden death from septicemia (Keene *et al.,* 1994). An outbreak of salmonellosis can have serious economic consequences on a farm as well as public health implications. Neonatal calves can present with septicemia (blood poisoning) which progresses rapidly to death within six to twelve hours. Initially, calves are dull and depressed and do not suck; diarrhea may be a terminal sign. Ingestion of colostrum from vaccinated dams (2 liters within the first 2-4 hours) is essential to reduce the risk of septicemia ((Whitman *et al.,* 2004).

Antibiotic resistance is a global public health concern. Almost 10 million people die per year due to antimicrobial resistance infections (Leelaporn *et al.,* 2003). Haphazard use of antibiotics and lack of knowledge are the most imperative variables for the rise, selection, and spread of antibiotic-resistant organisms in the environment. If such things happen continuously, it will bring a disaster to human being. At present, many of the antimicrobial agents are utilized in food animal production for controlling diseases and mostly used as growth promoter that is continuously disseminating in human food chain leads serious health problem in human and animals. These resistance elements can transfer to the people working on the farm directly from contaminated soil, water and milk to cause serious human health problems. Livestock manure contains microbial constituents which make it a potential source of pathogenic microorganisms for animals and human. About 151.3 million tons of fresh farm animal manure is produced in Bangladesh annually that is mostly used as biofertilizer in agriculture land (Integrated Livestock Manure Management Policy, 2005). Several bacterial pathogens such as *E. coli*, *Salmonella*, *Listeria*, *Coxiella*, and *Mycobacterium* have been recovered from manure that could be antibiotic-resistant and zoonotic in nature (Argudin M *et al*., 2017). These pathogens can enter into the food chain when manure used as fertilizer in agriculture for crop, vegetables, and fruit production to interferer consumers health.

Considering the above facts, the present study was conducted to investigate the occurrence and antibiogram assay of *E. coli* and *Salmonella* spp. in water used in cattle farms of selected areas in Dhaka City.

Objectives of the Study

- To isolate and identify the *E. coli* and *Salmonella* spp. from supplied water samples in different farm
- To know the prevalence of *E. coli* and *Salmonella* spp. in supplied water of study area
- Assessment of antibiotic resistance pattern of *E. coli* and *Salmonella* spp.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

Isolation, identification and antibiogram of *Escherichia coli* and S*almonella* spp. was performed using the information gained from the following related review of literature.

2.1 Isolation and identification of *E. coli* and *Salmonella* spp. from water sample

Escherichia coli was discovered by German bacteriologist Theodor Escherich in 1885 as a normal bacterial inhabitant of the intestine of healthy individuals and was named Bacterium coli commune (Escherich, 1988). It was later renamed in 1919 in his honor as *Escherichia coli* and is now classified as part of the Enterobacteriaceae family of gamma-proteobacteria (Feng *et al*., 2002; Kaper, 2005). They are gram-negative, nonspore forming, aerobic and facultative anaerobic bacilli which are either motile by means of peritrichous flagella or non-motile. They are closely related to other bacteria of the Enterobacteriaceae family like *Shigella* spp., *Citrobacter freundii*, and *Salmonella* spp. (Scheutz and Strockbine, 2005). *Salmonellae* are gram-negative, rod-shaped, non-spore forming, mainly motile enterobacteria. They are facultative anaerobes; belong to Enterobacteriaceae family having dimensions around 0.7 to 1.5 micro meter (diameters) and 2 to 5 micrometers (lengths) (Madigan *et al*., 2009). *Salmonellae* can grow at optimum temperature between 35°C to 37°C but growth has also been observed at temperatures ranging from 5°C to 47 °C. The optimum pH for growth is near 7.0, but growth may occur between pH 4.0 and pH 9.0 (Lanata *et a l*., 2003). *Salmonellae* produce hydrogen sulfide, decarboxylate lysine and ornithine, and do not hydrolyze urea. They are oxidase negative and catalase positive and can grow on citrate as the sole carbon source.

Monem *et al.* (1990) reported that sediments and soils are the potential habitats of *Salmonella* as the water currents and run off carries contaminated materials. *Salmonella* has been detected in soil samples collected from both agricultural and recreational areas (Thomason *et al.,*1977). *Salmonella* can survive and multiply for at least 1 year in this ecosystem (Davies *et al.* 1996; Thomason *et al.,*1977). The ability of *Salmonella* to grow and survive in diverse environment and ecosystem represent its cyclic lifestyle in host and nonhost environments.

Maringo *et al.* (1992) showed positive presence of pathogens i.e., *Salmonella* and indicator microorganisms (Fecal coliforms. *Streptococci*, *Clostridium perfringens*) in three aquatic environments affected by sewage discharges. Relationship between indicators and *Salmonella* depended mainly on source of fecal discharge. Survival capability of microorganisms in aquatic environment and percentage of *Salmonella* sp. was high even at low level of pollution. Results of study showed that there were no differences in survival rates between serotypes of *Salmonella. Salmonella* species also exhibited a similar persistence to *E. coli* in aquatic ecosystems.

Smith *et al.* (1994); Bogosian *et al*. (1996); Wcislo and Chrost, (2000); Guan and Holley, (2003); Sampson *et al*. (2006) reported that *E. coli* have a great capacity to survive for long periods in water. Studies indicate that several factors could increase or decrease the ability of *E. coli* to survive in a variety of aquatic conditions. These include biological factors like nutrient conditions and coliphage infection, physical conditions like varying water temperatures and chemical conditions like pH, dissolved solids and sediments.

Keene *et al.* (1994) reported that waterborne transmission of pathogenic *E. coli* is well documented for surface, recreational and drinking water. A simultaneous outbreak of bloody diarrhoea and hemolytic-uremic syndrome caused by *E. coli* O157 and bloody diarrhoea by *Shigella sonnei* due to swimming in a lakeside park in Portland, Oregon. The authors suggested that the pathogens survived in lake water and that their infectious dose was very low resulting in prolonged outbreak.

Bartram and Rees (2000); Rosas *et al.* (2006); Gupta *et al.* (2007) indicated that the use of untreated river water by people for domestic purposes including drinking, bathing, washing and laundry poses a high risk of infection assessed the source of pathogenic *E. coli* in beach water and sand at Lake Factors, associated with contamination of household drinking water among Tsunami and earthquake survivors in Indonesia.

Baudart *et al. (*2000) reported that *Salmonella* is constantly released into the environment from infected humans, farm animals, pets, and wildlife The organism is frequently isolated from surface and potable water (Chao *et al.* 1987; Cherry *et al.* 1972; Jyoti *et al*., 2010) which serve as bacterial reservoirs. *Salmonella* has the ability

to grow and survive in harsh conditions too as it can survive for 10 to 15 days in a septic system (Parker *et al*., 1982).

Zhao *et al*. (2001) documented that *E. coli* contaminated 19% of retail uncooked beef, of which 4% was *E. coli* O157:H7. *E. coli* O157:H7 was also detected in 1.5% of pork, 1.5% of poultry and 2% of lamb samples in Washington. Vegetables and fruit products like un-pasteurized fruit juice, vegetables, and salad ingredients such as lettuce, spinach, Alfa alfa and radish sprouts have also been associated with several disease outbreaks.

Whitman and Nevers (2003) studied Michigan Beach water and shore sand and noted high concentrations (1.1 × 104 ± 8.5 × 102 CFU/100 mL) of *E. coli* in foreshore sand that increased following gull activity. They also reported that with newly added sand to the shore, *E. coli* re-colonization took place in two weeks' time, making foreshore sand a continuous source of bacteria to beach water.

Kaper *et al*. (2004); Scheutz and Strockbine (2005) described that pathogenic *E. coli* include those types which cause enteric infections and those which cause extraintestinal infections. The virulence factors of pathogenic *E. coli* are encoded by genetic elements like plasmids, transposons and bacteriophages. These factors mostly promote colonization of the host, adherence or invasion of cells, evasion of host defences and disruption of host cell signaling pathways and include cell surface receptors, secreted enzymes and toxins.

Jagals *et al*. (2004) noted that when animals defecate on grazing fields or when infected manure is directly spread on land, the soil is contaminated with high levels of pathogenic *E. coli*. In the areas of inadequate or poor sanitation, open defecation in fields or on riverbanks could lead to build up of faecal matter in river basins. Faecal pollution can be introduced into surface and ground water from multiple sources including sewage overflows and agricultural runoff following heavy rains.

Mahalakshmi *et al.* (2011) reported high number of *E. coli* (5.9×10^4 CFU/mL) in water samples from the fishing harbour of Cuddalore, Tamilnadu, India which indicated the potential risk of contamination of aquatic life in the fishing dock. In 2007, two outbreaks of *E. coli* O157:H7 associated with recreational waters were reported in the United States. The first outbreak resulting in 11 persons becoming ill

was caused by a temporary inflatable waterslide at a California home. The second outbreak resulting in 31 cases was linked to an interactive fountain at a water park in Idaho. The investigation revealed that low chlorine levels (<0.5 mg/L) were responsible for the outbreaks (CDC, 2011).

2.2 Occurrence of *E. coli* and *Salmonella* spp.

Daoust *et al.* (1994) reported that ubiquitous distribution of *Salmonella* in the natural environment and its prevalence the physiological adaptability and virulence of this important bacterial pathogen and its serious impact on food industry predicate the need for continued vigilance control at all levels. Landerson *et al.* (1998), studied *S. enteritides* from 25 water samples and 38 food samples collected from Spain in 1985-96, these organisms were associated with poultry transmission, extra intestinal infections and sewage and environmental water.

Huerta *et al.* (2000) investigated a multifocal outbreak of diarrhoea in Israel, involving 175 Israel defence force soldiers and 54 civilians and detected enterotoxigenic *E. coli* (ETEC) in stool samples. Authors noted inadequate chlorination resulting in increase of *E. coli* concentrations in water samples collected from distribution lines. Kang *et al*. (2001), suggested faecal contamination of well water following rain to be responsible for two epidemics of acute watery diarrhoea in villages in North Arcot district, India. In the study, enteric pathogens were detected in 56.8 % and 60.3 % of 18 faecal specimens from the two villages. The isolation rates for EPEC and STEC strains were significantly higher during the epidemic.

Licence *et al.* (2001) traced the source of an outbreak of *E. coli* O157 in Highland Region of Scotland to an untreated and unprotected private water source. The isolates from water, sheep faeces and human stool collected from the affected area were similar in genetic profile underlining the route of transmission of *E. coli*. Olsen *et al.* (2002), studied a large outbreak of *E. coli* O157:H7 in Wyoming causing illness in 157 persons and reported that the outbreak was associated with drinking municipal water that was unchlorinated and contaminated with surface water.

Rangel *et al.* (2005) reported on epidemiology of *E. coli* O157:H7 outbreaks in the United States from 1982–2002 and showed that transmission route for 183 (52%) was food-borne and 31 (9%) was waterborne. Among waterborne outbreaks, 21 were from

recreational water and 10 from drinking water. **Hien *et al.* (2007)** detected diarrhegenic *E. coli* and other enteric pathogens in stool specimens of children with diarrhoea caused by the use of untreated wastewater in agriculture and aquaculture.

Shar *et al.* (2007) isolated total and faecal coliform bacteria from all samples of drinking water of Khairpur city having a surface reservoir as the primary source of water. The total coliform counts (log10 3.0-3.94 CFU/100 mL) and faecal coliform (*E. coli*) 17 counts (log10 1.46-2.47 CFU/100 mL) were found to be higher than the maximum microbial contaminant level (MMCL) established by WHO.

Garba *et al.* (2009) collected 180 water samples, 60 were from well, 60 from tap water and another 60 from packaged for the identification of *E. coli* and for estimating total coliform counts. After biochemical analysis of those samples indicated 63 confirmed presence of *E. coli*, prevalence for well water 45.5%, tap water 23.3% & packaged water was13.3%.

In a study by **Jokinen *et al.* (2010)** 342 samples were collected from surface water, 91 sample indicated *Salmonella* presence and 8 samples showed presence of *E. coli*. Ahmed *et al.* (2011) detected the prevalence of *Salmonella* spp. was 13.34% from drinking water in Dhaka City. Anera *et al.* (2014) collected 140 water samples to determine bacterial quality. Among these drinking water samples total coliforms bacteria, fecal coliforms bacteria and *E. coli* prevalence was 21.4%, 18.6% and 17.8%.

Moges *et al.* (2014) collected 60 samples from processed hospital waste water, they examined and found several types of bacteria among them 11.5% was *E. coli.* Shahidul *et al.* (2014); prevalence of *Salmonella* was detected 35% in case of restaurant water from Dhaka City.

Melissa *et al.* (2015) performed the study to conduct the microbiological parameters of environmental samples of fresh water from rivers of Curitiba and its metropolitan area in Parana State, Brazil. They detected the prevalence of *E. coli* was 19.43%.

Khan *et al.* (2016) observed 100 samples & out of them 17 samples was positive for total coliform bacteria. Kumarasingam *et al.* (2016) studied 140 waste water samples

and recycle them for 2 hours, isolated several bacteria and the prevalence of *E. coli* was 16.42%.

Osvalda *et al*. (2017) collected 182 water samples which was used for irrigation purpose and the prevalence of *E. coli* and *salmonella* spp. was 77.5%.

in a study by **Mahagamage *et al.* (2020)** 72 ground water sample were collected and 45 Surface water samples were examined. Almost all the samples were contaminated with *E. coli* and in case of ground water 17 samples were present *Salmonella* spp. and 26 samples were present *salmonella* spp. for surface water.

Aftab *et al.* (2020) observed 18 samples of bottled and jar water from Dhaka City and *E. coli* was present in 8 samples and the prevalence was 44.44%. **Jamil *et al.* (2020)** observed 425 primary school water samples from ten districts of Sindh, Pakistan. They had used quantitative microbial risk assessment technique to detect the possibility of infection among school children by the consumption of that water. They found around half of the water samples from those schools were contaminated with *E. coli* (49%) & *Salmonella* spp. (53%).

2.3 Antibiotic resistance pattern of *E. coli* and *Salmonella* spp.

Chattopadhya *et al.* (2001) observed that all the *E. coli* (STEC) isolates (12 animals, 1 human and 4 food samples) from a total of 876 samples (330 animals,184 humans and 362 food samples) were uniformly sensitive to common antibiotics, except tetracycline, dicloxacillin, erythromycin, cephalexin and lincomycin.

Tiwari and Adhikari (2001) studied pond water and drinking-pot water samples from Kathmandu valley during four different seasons and the prevalence and resistance to 21 antibiotics were determined. In the study, 37 *E. coli* isolates were obtained and among them 32 % were resistant to one or more antibiotics showing high contamination of water sources

Leelaporn *et al.* (2003) performed antimicrobial susceptibility tests of *E. coli* isolates in Bangkok, by disc diffusion method. All the isolates were found susceptible to cefaclor, ceftriaxone, imipenem, netilmicin, norfloxacin, ciprofloxacin, nalidixic acid. More than 90% of the isolates were susceptible to cefdinir, gentamycin, neomycin and

chloramphenicol. Resistance rates to ampicillin, co-trimoxazole and tetracycline were 17, 39, and 65 percent, respectively.

Su *et al.* (2004) reported that various *Salmonella* serovars are resistant to conventional antibiotics such as ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole and other newer antibiotics (quinolones and extended-spectrum cephalosporins) have been reported with increasing frequency in many areas of the world.

Kaper *et al*. (2005) reported that during the last decade, antibiotic resistance and multi drug resistance of *Salmonella* spp. have increased a great deal, especially in developing countries with an increased and indiscriminate use of antibiotics in the treatment of humans and animals.

Matasejea *et al.* (2009) studied environmental water (recreational beach, drinking water) samples from Canada for cefoxitin resistant *E. coli*. Of 142 cefoxitin resistant *E. coli* strains isolated from water sources; 65 isolates were MDR. Isolates showed high resistance to amoxicillin/clavulanic acid, ampicillin and ceftiofur other than cefoxitin.

Ibekwe *et al.* (2011) investigated the antimicrobial resistance pattern of *E. coli* isolated from small channels arising from middle Santa Ana River in Southern California and identified the source of contamination to be that of humans and animals. Twenty-four percent of the 600 isolates exhibited resistance to more than one antimicrobial agent. Most multiple resistances were associated with inputs from urban runoff and involved the antibiotics rifampicin, tetracycline, and erythromycin.

Rajabi *et al*. (2011) showed that total 78 (2.0%) *Salmonella* serotype isolated from 3,980 blood culture samples, in which 47 (60.3%) were *S. typhi* and 31 (39.7%) were *S. paratyphi*. Isolates were from all age group median age being the 25 years. Among the tested antibiotics *S. typhi* was susceptible towards Ciprofloxacin (100%) followed by Gentamicin (97.9%), Ofloxacine (95.7%), Ceftriaxone (95.7%) and Chloramphenicol (93.6%). In case of *S. paratyphi* most of the tested antibiotics showed high percentage of susceptibility and least susceptible antibiotic for *S. paratyphi* was Ampicillin (25.8%). Three isolates of *S. typhi* showed multidrug resistance.

CHAPTER 3

MATERIALS & METHODS

CHAPTER 3

MATERIALS AND METHODS

This study was conducted at the of Microbiology & Parasitology Department of Animal Science and Veterinary Medicine faculty of Sher-e-Bangla Agricultural University (SAU), Sher-e-Bangla Nagar, Dhaka 1207, during the period of October, 2020 to April, 2021.

3.1 Materials and chemicals

3.1.1 Glass wares and others instrument

- ➢ Collecting vial
- ➢ Test tube
- ➢ Test tube holder/ rack
- ➢ Conical flask
- ➢ Spirit lamp
- ➢ Cotton, foal paper
- ➢ Petri dish
- ➢ Eppendorf tube
- ➢ Pipette
- ➢ Micropipette
- ➢ Measuring cylinder
- ➢ Electric balance machine
- ➢ Electric stirrer
- ➢ Glass spreader
- ➢ Streaking loop
- ➢ Incubator
- ➢ Laminar air flow
- ➢ Refrigerator

3.1.2 Chemical reagents

- ➢ PBS (Phosphate buffer solution)
- ➢ 70% Alcohol
- ➢ Distilled Water
- ➢ 3% Hydrogen per Oxide (H_2O_2)
- ➢ Normal saline solution
- ➢ 50% Buffered Glycerol Saline
- ➢ Other common laboratory chemicals

3.2 Sample collection site

This study was conducted to investigate the prevalence of bacteria in the water used in different cattle farm in Dhaka City. A total of 100 drinking water samples were collected from the waterer of different cattle farms from seven different areas of Dhaka during the period from October, 2020 to April, 2021 (Table 1). Collected samples were immediately transported on ice to the laboratory of the Department of Microbiology & Parasitology, Sher-e-Bangla Agricultural University for analysis. The samples were directly transferred in an icebox to the laboratory for further preparation and examination.

Table 1. Number of water samples collected from different cattle farms of Dhaka city

SL. No.	Sample Collected Area	No. of Samples
01	Adabor	15
02	Dhaka Uddan	15
03	Bosila	15
04	Hazaribagh	15
05	Mirpur	15
06	Keraniganj	15
07	Gabtoli	10
Total Samples		100

3.3 Duration of the experiment

Water samples were collected from 7 sites of Dhaka city and the study was performed at the laboratory of the Department of Microbiology & Parasitology, Sher-e-Bangla Agricultural University, Dhaka-1207. The duration of the experiment was from October, 2020 to April, 2021.

3.4 Experimental design

The whole experimental design is accomplished into two steps. The first step included isolation of the bacteria from cattle farm water and identification of *Salmonella* spp. and *E. coli* by cultural and morphological characteristics. The second step included the study of response of the isolated bacteria against commercially available antibiotic discs. Fresh water samples were collected from the different areas of Dhaka city. Then, they were cultured in Nutrient broth for multiplication. Primary growths of bacteria of each collected sample were performed in NB. Then 10-fold dilution was prepared and spread on Nutrient agar medium for total viable count (TVC) and on MacConkey agar for total coliform count (TCC). Subcultures were grown on SS (*Salmonella –Shigella*) agar, MacConkey agar and EMB (Eosin Methylene Blue) agar media for obtaining pure culture of the isolated organisms. After determining cultural character, these pure cultures of the organisms were subjected to staining and morphological examination for identification of organisms. Then Gram's staining and biochemical tests were performed. Finally, the isolated organisms were subjected to antibiogram study to observe the resistant characteristics of organism on some specific antibiotic disk.

The following is a flow chart of representing design of the experiment

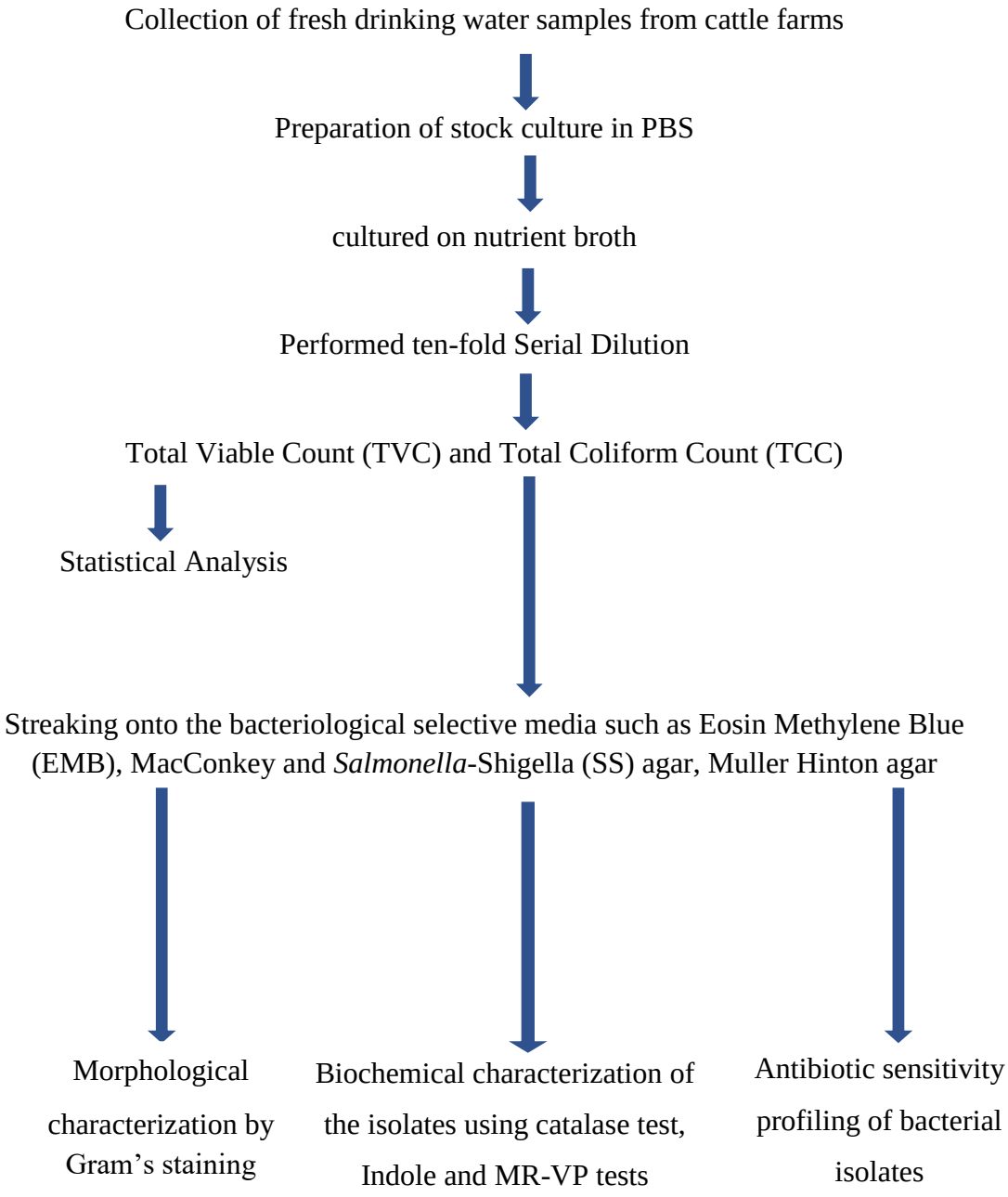

Figure 1: Schematic illustration of the experimental design

3.5 PBS (Phosphate Buffered Saline) Solution Preparation

PBS (Phosphate Buffered Saline) solution prepared by using-

- ➢ 0.2 gm. of potassium chloride
- ➢ 2.89 gm. of disodium phosphate
- ➢ 8 gm. of sodium chloride and
- ➢ 0.2 gm. of potassium hydrogen phosphate

Those were mixed in 1000 ml of distilled water. With an electric magnetic stirrer, the solution was mixed and heated to dissolve properly. Then sterilized by autoclaving at 121^0C temperature at 15 lbs. / $inch^2$ for 15 minutes. Then stored it for further use.

3.6 70% Alcohol Preparation

For preparation of 70% alcohol, pure ethanol (100% ethanol) was used. Through a measuring cylinder 700 ml pure ethanol was mixed with 300 ml of distilled water.

3.7 50% Buffered Glycerol Saline Preparation

For preparation of 50% Buffered Glycerol Saline, 8.3 gm buffered glycerol saline base was added in 700 ml demineralized water. Then added 300 ml glycerol in it. Then heated it to dissolve completely. After mixing well, it was dispensed in tubes, which was screw capped or in containers. Autoclaved for sterilization at 121^0 C temperatures at 15 lbs. / inch pressure for 15 minutes.

3.8 Media used for bacterial observation

3.8.1 Solid Media

3.8.1.1 Nutrient Agar Media

For preparation of Nutrient agar media, in a conical flask 2.8gm powder of nutrient agar was dissolved in 100 ml distilled water and boiled to dissolve completely through electric stirrer. Then it was sterilized by autoclaving at 121^0 C temperature at 15 lbs. / $inch^2$ for 15 minutes. After autoclaving media was poured into Petri dishes

for solidification and the quantity of media for medium size Petri dish was 10ml/ Petri dish and for large size the amount was 15ml/ Petri dish. Then inoculated and incubated at 37^0 C temperature for overnight.

3.8.1.2

MacConkey Agar Media

To prepare MacConkey agar media, 51.53 gm powder of MacConkey agar was dissolved in a conical flask containing 1000 ml distilled water and boiled to dissolve completely. Then sterilized by autoclaving at 121^0 C temperature at 15 lbs./ $inch^2$ for 15 minutes. After autoclaving media was poured into Petri dishes for solidification and the quantity of media for medium size Petri dish was 10ml/ Petri dish and for large size the amount was 15ml/ Petri dish. Then inoculated and incubate at 37^0 C temperature for overnight.

3.8.1.3 *Salmonella-Shigella* (SS) agar media

To prepare *Salmonella-Shigella* (SS) agar media, 63.02 gm powder of SS agar dissolved in a conical flask containing 1000 ml distilled water and heated to dissolve completely. Then sterilized by autoclaving at 121^0 C temperature at 15 lbs. / $inch^2$ for 15 minutes. After autoclaving media was poured into Petri dishes for solidification and the quantity of media for medium size Petri dish was 10ml/ Petri dish and for large size the amount was 15ml/ Petri dish. Then inoculated and incubate at 37^0 C temperature for overnight.

3.8.1.4 Eosin Methylene Blue (EMB) agar media

For preparation of EMB agar media, in a conical flask 35.96 gm EMB agar powder was dissolved in 1000 ml distilled water and boiled to dissolve completely. Then sterilized by autoclaving at 121^0 C temperature at 15 lbs. / $inch^2$ for 15 minutes. After autoclaving media was poured into Petri dishes for solidification and the quantity of media for medium size Petri dishes was 10ml/ Petri dishes and for large size the amount was 15ml/ Petri dishes. Then inoculated and incubate at 37^0 C temperature for overnight.

3.8.2 Liquid Media

3.8.2.1 Nutrient Broth

Nutrient broth is a liquid media used as a primary media for inoculation and initial growth of bacteria occurred here. For preparation of Nutrient broth media, in a conical flask 13.0gm nutrient broth powder dissolved in 1000 ml distilled water and heated to boil and to dissolve completely. Then sterilized by autoclaving at 121^0 C temperature at 15 lbs./ $inch^2$ for 15 minutes. After autoclaving media was allowed to be cool. After cooling it poured into the previously sterilized test tube and the quantity is 5ml/test tube. 100 micro litter sample was added into each test tube and incubated at 37^0c temperature for 24 hours.

3.8.2.2 Methyl-Red Voges-Proskauer (MR-VP) broth

For preparation of MR-VP broth media, in a conical flask 17gm broth powder dissolved in 1000 ml distilled water and heated to boil and to dissolve completely. Then sterilized by autoclaving at 121^0 C temperature at 15 lbs./ $inch^2$ for 15 minutes. After autoclaving, allowed to be cool. After cooling it poured into the previously sterilized test tube and the quantity was 2ml/test tube. Then it was allowed to incubate at 37^0 C for 72 hours.

3.8.2.3 Muller Hinton agar (MHA)

38 gm of dehydrated Muller Hinton Agar Medium was suspended in 1000 ml cold distilled water and boiled to dissolve the medium completely. The solution was then sterilized by autoclaving at 121°C and 15 lbs. pressure per sq. inch for 15 minutes. The autoclaved materials were allowed to cool to a temperature of 45°C in a water bath and distributed to sterile Petri dishes properly. After complete solidification, Petri dishes were placed in an incubator for 24 hours at 37° C to check its sterility and then placed in a refrigerator at 4°C for future use.

3.9 Materials required for antibiogram study

3.9.1 Muller Hinton Agar (MHA)

Muller Hinton Agar plates were specially used for the antibiogram study test (Himedia, India).

3.9.2 McFarland standards

McFarland standards are used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within the standard range.

3.10 Bacteriological test

3.10.1 Collection & transportation

Water samples were collected from following area by using collecting vial or conical flask & transported aseptically to the microbiology laboratory in SAU campus. Samples were used for observation within 24 hours after collection.

3.10.2 Preparation of primary inoculum

100 micro litter of each sample was added into 5ml Nutrient broth containing test tube. This was then incubated at 37^0 C temperature for overnight.

3.10.3 Preparation of ten-fold serial dilution and spread plate method

For preparation of serial dilution, a series of Eppendorf tube was taken to the tube holder. 9 ml PBS solution was taken into each tube. 1 ml sample was then added to the first tube containing 9 ml PBS. Mixed it properly. Then 1 ml dilution discarded from the first tube and poured into second one. Again, mixed it properly. By this following method this was continued to the last one. From the last Eppendorf tube, 1 ml dilution was discarded. Each dilution of each sample was then spread over the Nutrient Agar & MacConkey Agar media by spread plate method. A glass spreader with smooth edge was used for this spread plate method. 0.1 ml mixture was transferred into each Petri dish containing EMB & MacConkey Agar media. Glass spreader was sterilized by 70% alcohol and burned it in Bunsen burner before using it for spreading in each media containing Petri dish. After spreading, the Petri dishes incubated at 37^0 C temperature for overnight. Then each plate was observed & 30-300 colonies containing plates were counted, more or less was discarded.

3.10.4 Isolation & streak plate method

The colony was then isolated and inoculated by streaking into different media to observe the growth of the bacteria in different media containing plate. Then incubated at 37^0 C temperature for 24 hours.

3.10.5 Gram's staining procedure & microscopic examination

For performing gram's staining procedure, a small colony was picked up with a sterile loop in a glass slide and smeared. Then heating gently to fixed on it. Applied crystal violet as a primary stain into the smear on glass slide & allowed to stain for two minutes. Then washed out with running tap water. Followed by few drops gram's iodine as a mordant was added and then washed out with running tap water. After washing rapidly added alcohol for decolorization. After this added safranin as a counterstaining and kept it for two minutes. Then again washed out with running tap water. Followed by blotted and air dried to examined under microscope (100X) by using immersion oil.

3.11 Biochemical analysis

3.11.1 Catalase Test

For catalase test, culture was picked up with a sterile loop in a sterile glass slide. The test was performed by using 3% H_2O_2. Culture was taken to the agar plate with a sterile loop and a drop of 3% H_2O_2 was mixed on a clean sterile glass slide. Bubble was formed within a few seconds after adding H_2O_2 & it became positive. In case of negative there had no change after adding H_2O_2 with the colony.

3.11.2 MR Test

After preparation of medium 5ml/ test tube was taken and added the inoculum into this test tube. Incubated for 72 hours at 37^0c temperature. After incubation one drop of methyl red solution was then added in this test tube. After mixing, red color was indicated positive result & yellow color or no color change indicated negative result.

3.11.3 VP Test

2 ml of sterile glucose peptone water was inoculated with the 2 ml of test organisms. It was incubated at 37^0C for 48 hours. A very small amount of creatine was added and mixed. 3 ml of sodium hydroxide was added and shacked well. The bottle cap was removed and left for an hour at room temperature. It was observed closely for the slow development of a pink color for positive cases. In case of negative reaction there was no development of pink color.

3.11.4 Indole test

The organisms kept in a test tube which has 3 ml of peptone water containing tryptophan and cultured at 37º C for 48 hours in incubator. After incubation 1 ml of diethyl ether was added then shaked and allowed to stand until the ether rises to the top. Then gently added 0.5 ml of Kovac's reagent by running down the side of the test tube. So that it formed a ring in between the medium and the ether layer. Observation was performed for the development of ring. Development of a brilliant red colored ring indicated indole production. In negative case, no development of ring (Chesbrough, 2006).

3.12 Antibiogram study

The disc diffusion method was used to detect antimicrobial susceptibility according to the recommendation of Clinical and Laboratory Standards Institute (CLSI) (formerly National Committee for Clinical Laboratory Standards, CCLS: 2016). Antimicrobial drug susceptibility against 5 commonly used antibiotics were performed by disc diffusion or Kirby–Bauer method (Bauer *et al.,* 1966). The procedure of disc diffusion method is presented below:

i. One well isolated colony was selected from the SS and EMB agar plate.

ii. Colony was touched with a sterile loop and streaked onto nutrient agar and incubated overnight at 37°C.

iii. 4 or 5 well isolated colonies were transferred into a tube of sterile physiological saline and vortex thoroughly.

iv. The bacterial suspension was compared with 0.5 McFarland standard. The comparison was made by viewing this tube against a sheet of white paper on which blacklinesweredrawn.

v. A sterile cotton swab was dipped into the bacterial suspension. The excess fluid of swab was removed by pressing firmly against the inside of the tube just above the fluid level.

vi. The swab was streaked over the entire surface of Mueller-Hinton agar (Himedia, India) medium three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculums.

vii. The antimicrobial discs were placed individually using sterile forceps and then gently press down onto the agar.

viii. The plates were inverted and incubated at 37°C temperature for overnight. After incubation the diameter of the zone of complete inhibition (including diameter of the discs) was measured in millimeters with a ruler.

3.12.1 Interpretation of the results

After the discs are placed on the plate, the plates were inverted and incubated at 37℃ for 8 to 12 hours following which the diameter of the zones of complete inhibition (including the diameter of the disc) was measured and recorded in millimeters. The measurements were made with a ruler on the under surface of the plate without opening the lid. The zones of growth inhibition were compared with the zone-size interpretative table provided by Clinical and Laboratory Standards Institute (CLSI, 2016) (Table No.2). Antimicrobial testing results were recorded as susceptible, intermediate and resistant according to zone diameter interpretive standards provided by CLSI (2016).

Table 2: The zone-size (mm) of *E. coli* and *Salmonella* spp. interpretative table provided by Clinical and Laboratory Standards Institute (CLSI, 2016).

Antimicrobial agents	Disc Concentration (µg)	Resistant (mm)	Intermediate (mm)	Sensitive (mm)
Streptomycin	10	≤15	16-21	≥22
Ciprofloxacin	5	≤ 20	21-30	≥ 31
Ampicillin	10	≤12	13-19	≥20
Tetracycline	10	≤11	12-14	≥15
Gentamycin	30	≤12	13-14	≥15

CHAPTER 4

RESULT & DISCUSSION

CHAPTER 4

RESULTS & DISCUSSION

The results presented below demonstrated the isolation and identification of bacterial isolates from water samples of different cattle farms in and around Dhaka city. The results also indicate the sensitivity and resistance pattern of the isolates to five different antibiotics.

4.1 Bacteriological examination

At first, all the collected water samples were incubated in nutrient broth. Then, cultured in MacConkey agar media & Nutrient agar media to count the TCC of Coliform bacteria. The stock sample from Nutrient broth cultured in EMB agar media for isolation of *E. coli* and before counting the *E. coli* it was confirmed by biochemical test. The colony also cultured in *Salmonella- Shigella* Agar media and Eosin Methylene Blue Agar media.

4.2 Physical examination

Physical examination was done by necked eye to detect the color, odor, turbidity, PH level (detected with paper stripes), specific gravity & presence of any foreign particles in each sample. If any difficulties found, they were discarded for further analysis.

Table 3: Physical parameter observation after collection of all samples

Parameters	Result
Color	Colorless
Odor	Odorless
Turbidity	Nil
P^H	Near about 7
Any foreign particles	Not visible

When all the physical parameters were favorable, the further study was continued otherwise the samples were discarded.

4.3 Culture in nutrient broth

The entire sample cultured in nutrient broth showed turbidity after incubated overnight which confirms the growth of bacteria.

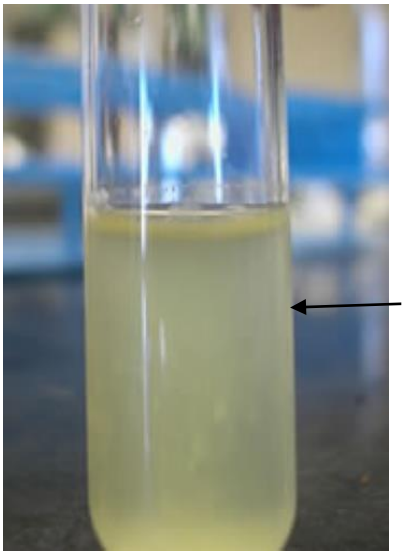

Figure 2: Turbidity grow in nutrient broth media

4.4 Determination of TVC and TCC

Total viable count (TVC) of cattle farm water samples collected from the different areas of Dhaka city are presented in (Table 4). The bacterial load was highest at Gabtoli (3.51 ± 2.61) $\times 10^5$ (cfu/ml) followed by Bosila (2.52 ± 2.66) $\times 10^5$ (cfu/ml), Mirpur (4.11 ±2.86) $\times 10^4$ (cfu/ml). The lowest bacterial density at Keranigonj (5.02 ± 2.41) $\times 10^3$ (cfu/ml).The total coliform count (TTC) of cattle farm water samples collected from the different areas of Dhaka city are presented in (Table 4). The bacterial load was highest at Gabtoli (3.47 ± 3.11) $\times 10^5$ (cfu/ml) followed by Hazaribagh (6.61 ±2.86) $\times 10^4$ (cfu/ml) and Keranigonj (5.37 ± 2.81) $\times 10^4$ (cfu/ml). The lowest bacterial density was at Mirpur (2.85 ±1.67) $\times 10^3$ (cfu/ml).

Table 4: Total Viable Count (TVC) (Cfu/ml) and Total Coliform Count (TCC) (Cfu/ml)

from the isolated water samples

Serial Number	Area of sample collection	Number of Sample Tested	Total Viable Count (TVC)(Cfu/ml) (Mean ± Standard Deviation)	Total Coliform Count (TCC)(Cfu/ml) (Mean ± Standard Deviation)
1	Adabor	15	$(3.86 \pm 2.16) \times 10^4$	$(3.29 \pm 0.88) \times 10^4$
2	Dhaka Uddan	15	$(3.17 \pm 2.32) \times 10^4$	$(3.02 \pm 2.41) \times 10^3$
3	Bosila	15	$(2.52 \pm 2.66) \times 10^5$	$(4.11 \pm 3.17) \times 10^4$
4	Hazaribagh	15	$(8.73 \pm 1.99) \times 10^3$	$(6.61 \pm 2.86) \times 10^4$
5	Mirpur	15	$(4.11 \pm 2.86) \times 10^4$	$(2.85 \pm 1.67) \times 10^3$
6	Keraniganj	15	$(5.02 \pm 2.41) \times 10^3$	$(5.37 \pm 2.81) \times 10^4$
7	Gabtoli	10	$(3.51 \pm 2.61) \times 10^5$	$(3.47 \pm 3.11) \times 10^5$

4.4.1 Bacterial colony formed on Nutrient agar

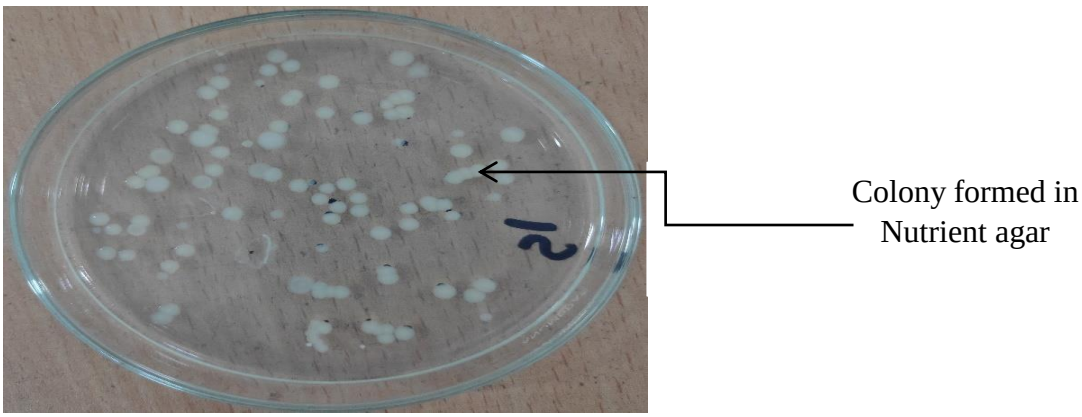

Figure 3: Colony count from Nutrient agar for estimation of TVC (Ten-fold dilution method)

TVC (total viable count) was performed by enumerating of the inoculated 0.1 ml stock culture sample from each dilution into nutrient agar (NA) using the spread plate

method. The method was performed according to the book named Manual of veterinary microbiology by Gustave Mosselman .

4.4.2 Culture in MacConkey agar media

In MacConkey agar media reddish to pinkish, whitish, dark centered brown colored colony was found which are characterized for coliform bacteria. Reddish, pinkish colony indicated the lactose fermenting coliform where whitish and brown color colony indicated the non-lactose fermenting coliform bacteria.

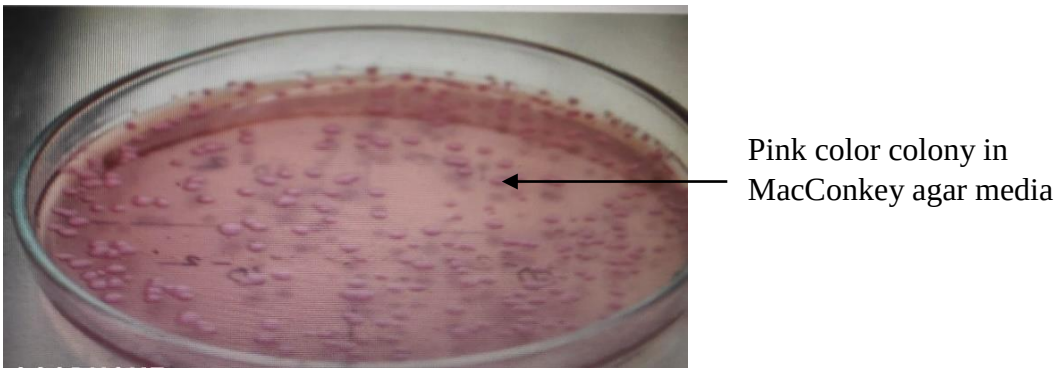

Figure 4: Total coliform count by ten-fold dilution method

4.5 Identification of *E. coli* in media

4.5.1 In MacConkey agar media:

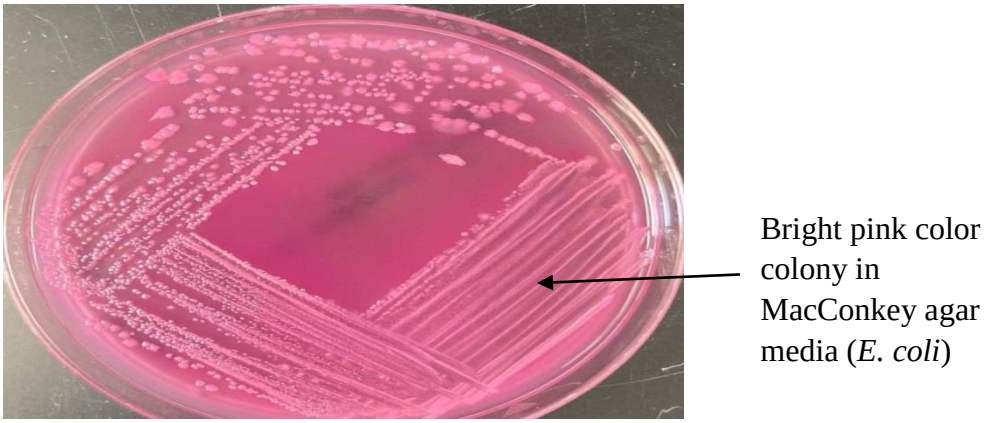

Figure 5: Pink color colony in MacConkey Agar Media

4.5.2 In EMB agar media:

E. coli formed greenish colony with metallic sheen Cultured in EMB agar by streak plate method after incubation for 24 hours at 37^0 c temperature.

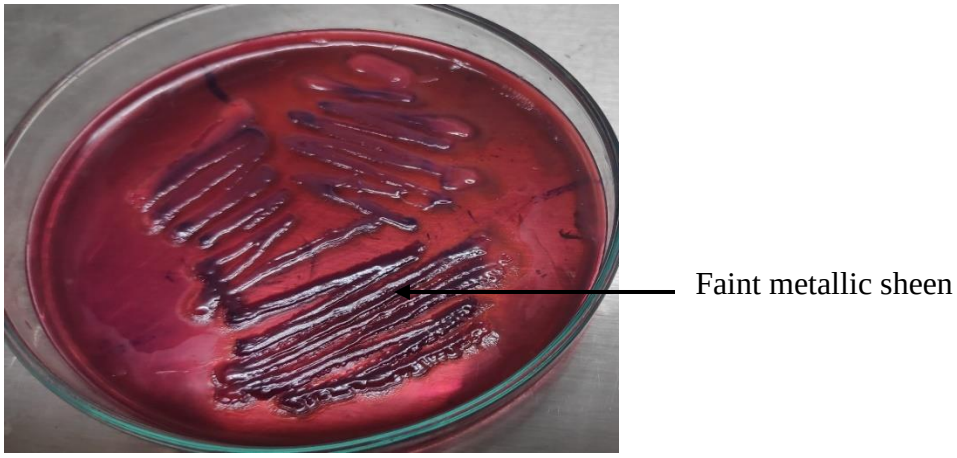

Figure 6: Greenish red color colony with faint metallic sheen(EMB agar)

4.6 Gram's staining and microscopic observation of *E. coli*

After gram's staining observed under light microscope in case of *E. coli* observation, it indicated gram-negative, rod shaped, pink color and organism arranged as single or paired.

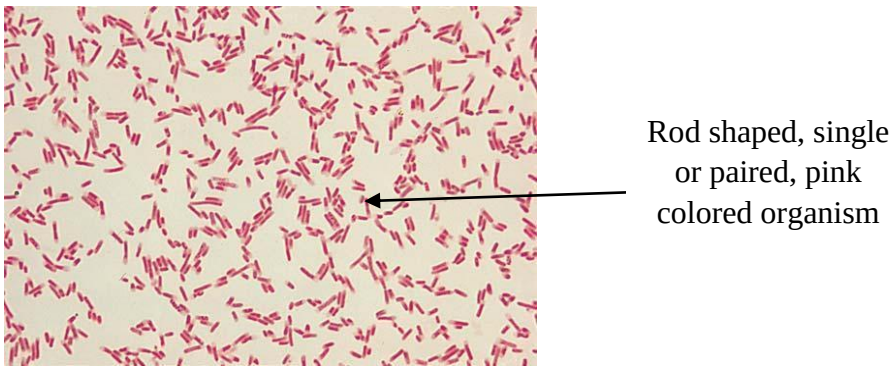

Figure 7: *E. coli* under microscope (100X)

4.7 Biochemical test to detect *E. coli*

4.7.1 Catalase test

In catalase test, bubble formation occurred within a few seconds after adding 3% H_2O_2 solution indicated the positive test for *E. coli.*

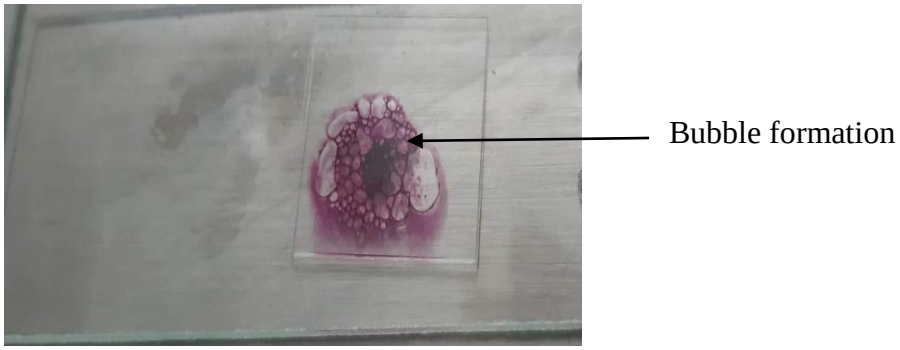

Figure 8: Bubble formation in catalase test

4.7.2 MR-VP Test

After preparation of MR and VP medium, added inoculum into test tube.Then Incubated for 72 hours. After incubation, one drop of methyl red solution was added in each test tube. After that, red color was indicated MR positive result & no colour no color change indicated VP negative result.

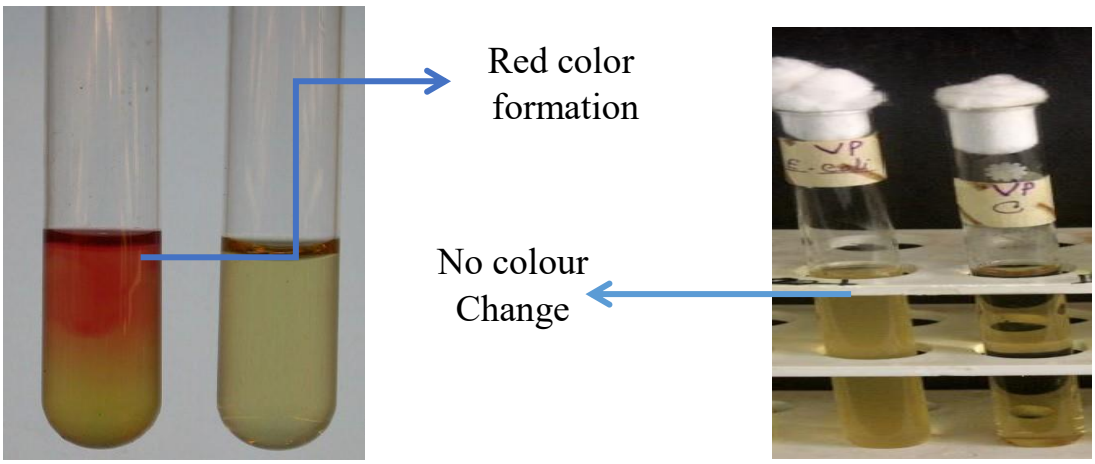

Figure 9: MR-VP Test

4.7.3 Indole test

After added Kovac's reagent, it forms a ring in between the medium and the ether layer. Development of a brilliant red colored ring indicated indole production. In negative case there is no development of red color (Cheesbrough, 2006).

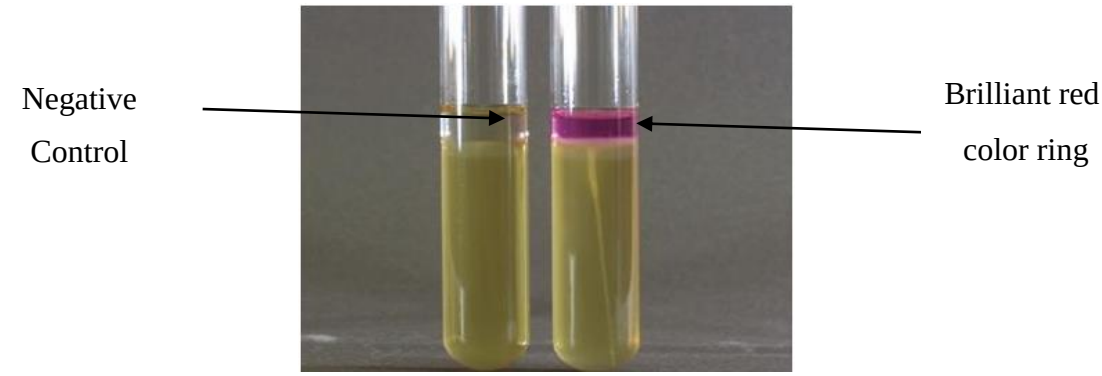

Figure 10: Indole test is positive for *E. coli*

Isolation and identification results of the study indicated that the selected samples contained gram-negative and motile organisms of *E. coli. Colony* characteristics of *E. coli* in two different agar media was prominent and all the *E. coli* isolates were able to produce characteristic metallic sheen colony on the EMB agar, bright pink colony on MacConkey agar. In Gram's staining, the morphology of the isolated bacteria exhibited pink, small rod-shaped gram-negative bacilli. Similar result was found in a previous study by (Rawal *et al.,* 2013) from tap water. These findings were supported by several authors such as (Buxton and Fraser, 1977), (Freeman, 1985) and (Jones *et al.,* 1987). The results of Catalase, MR (Methyl Red) and indole test of *E. coli* isolates were positive but VP (Vosges Proskauer) test was negative as reported by (Buxton and Fraser, 1977).

4.8 Identification of *Salmonella* spp.

4.8.1 On SS Agar

Salmonella spp. on SS (*Salmonella-5Shigella)* agar was indicated by smooth, circular, black color colonies.

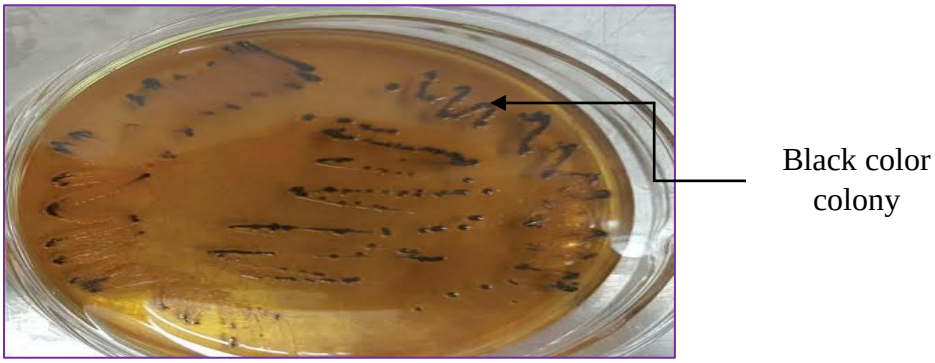

Figure 11: Black colony in SS Agar

4.8.2 On MacConkey Agar Media

Salmonella spp. on MC agar media produced Red to pink-white color colonies surrounded by red zones.

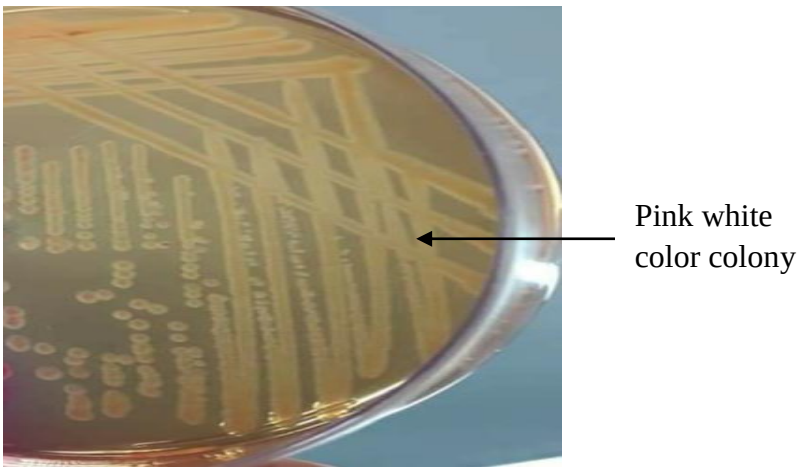

Figure 12: Red to Pink color colony in MacConkey Agar Media

4.9 Gram's staining and microscopic observation for *Salmonella* spp.

After gram's staining observed under light microscope. In case of *Salmonella* observation, it indicated gram negative, rod shaped, pink color & organism arranged as single or paired.

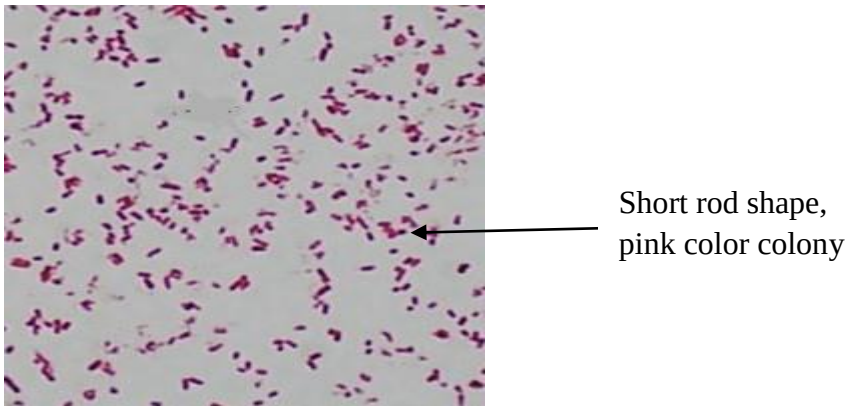

Figure 13: Microscopic observation of *Salmonella* (100X)

4.10 Biochemical test to detect *Salmonella sp.*

4.10.1 Catalase test

In catalase test, bubble formation within a few seconds after added 3% H_2O_2 solution indicated the positive test for *Salmonella* spp.

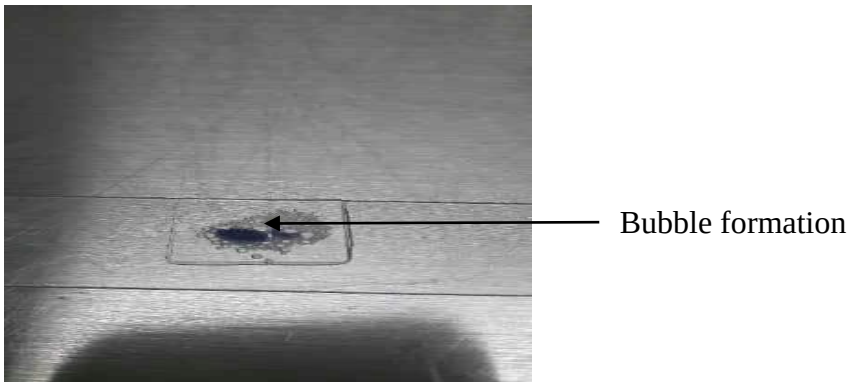

Figure 14: Catalase Test (positive)

4.10.2 MR-VP Test

After preparation of media, added inoculum into each test tube.Then Incubated for 72 hours. After incubation one drop of methyl red solution was added in each test tube. After that red color was indicated positive result & yellow color or no color change indicated negative result.

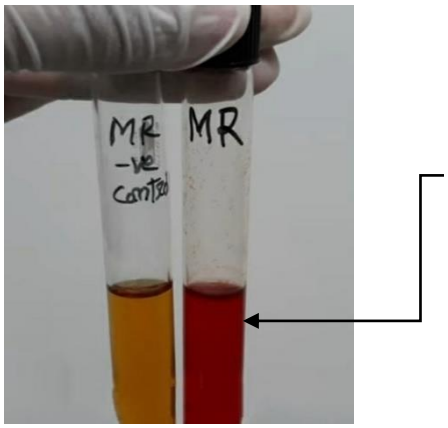

Figure 15: MR Test (Positive)

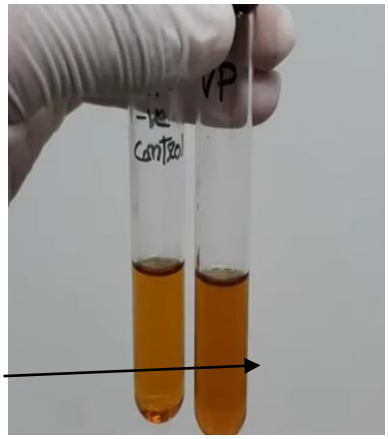

Figure 16: VP Test (Negative)

4.10.3 Indole Test

After added Kovac's reagent, it forms a ring in between the medium and the ether layer. Development of a brilliant red colored ring indicated indole production. In negative case there is no development of red color (Cheesbrough, 2006).

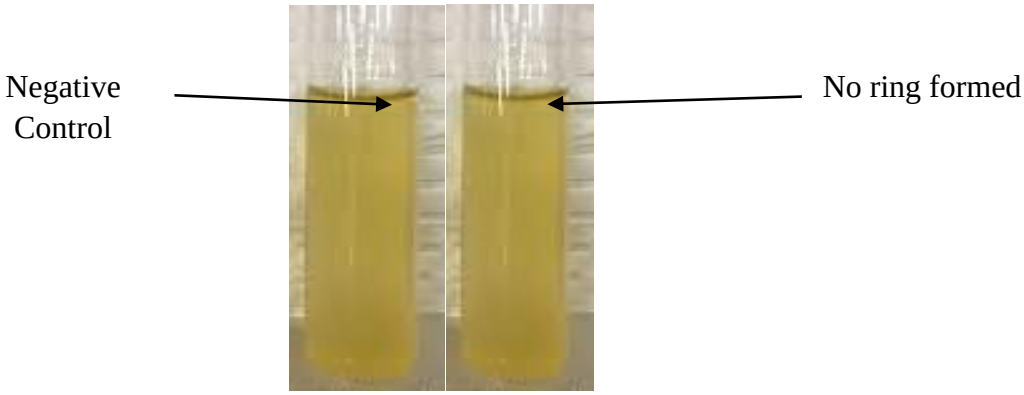

Figure 17: Indole Test (negative)

All the *Salmonella* isolates were able to produce Red to pink-white colonies surrounded by brilliant red zones in MacConkey agar media & Colonies with black

centers in SS agar. In Gram's staining, the morphology of the isolated bacteria exhibited pink, small rod-shaped Gram-negative bacilli. These findings were supported by several authors such as Buxton and Fraser (1977), Freeman (1985) and (Jones *et al.*, 1987). The result of Indole test for *Salmonella* was negative (formation of yellow ring), catalase test was positive, MR (Methyl Red) test was positive & result of VP (Vosges Proskauer) test was negative which satisfy the statement of Buxton and Fraser (1977).

4.11 Prevalence of microorganism in water samples

4.11.1 Prevalence percentage of *E. coli* & *Salmonella* spp.

Table 5: Prevalence of microorganism in water samples from different region

SL. No.	Sample Collected Area	No. of Samples	No. of *E. coli* positive	No. of *Salmonella* spp. positive
01	Adabor	15	7 (46.67 %)	5 (33.33%)
02	Dhaka Uddan	15	6 (40%)	4(26.67%)
03	Bosila	15	9 (60%)	7(46.67%)
04	Hazaribagh	15	6 (40%)	6(40%)
05	Mirpur	15	8 (53.33%)	6(40%)
06	Keraniganj	15	5(33.33%)	4(26.67%)
07	Gabtoli	10	7 (70%)	5(50%)

Table 6: Prevalence of microorganism in water samples

Parameter	No. of Samples Investigated	No. of Samples containing organism	Prevalence of *E. coli*	Prevalence of *Salmonella* spp.
TVC (Total Viable Count)	100	100	46%	37%
TCC (Total Coliform Count)	100	43		

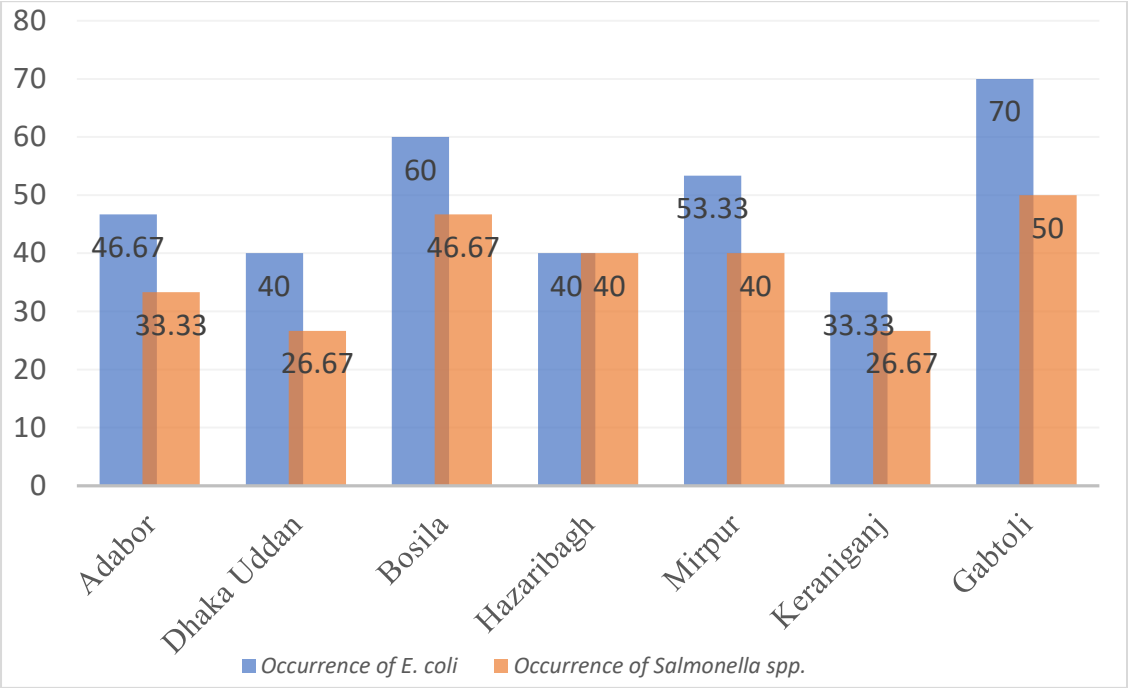

Figure 18: Graphical representation of occurrence (%) of *E. coli* and *Salmonella* spp. in water

In this study, the occurrence of *E. coli* in the water samples was 46% and 37% for *Salmonella* spp. 46 samples among 100 samples were infected with *E. coli*, 37 samples were infected with *Salmonella* spp. (among 100 samples) and rest samples were unidentified. Presence of *E. coli* in water indicates that the source is subject to recent fecal contamination of animals or human origin (Ashbolt *et al.*, 2001). The water sources considered for this study were from the different cattle farm located at Adabor, Dhaka Uddan, Bosila, Hazaribagh, Mirpur, Keraniganj, Gabtoli in Dhaka city. The rate of isolation of *E. coli* was the highest (70%) for Gabtoli followed by Bosila (60%), Mirpur (53.33 %) and least for Keraniginj (33.33%). This could be due to the nature of these sources. In present study, all water sampling sites showed the presence of microorganisms. The overall incidence of *E. coli* was 46%. Nguendo-Yongsi (2011), obtained similar results for piped drinking water supplies of a Sub-Saharan community in Yaounde. They isolated 1242 enteric bacteria from all the

samples of which 51.5 % isolates were *E. coli* strains making more than 90 % of the drinking water sources of poor standards as per WHO.

In another supporting study, Prasai *et al*. (2007), investigated the quality of various water sources of Kathmandu valley and isolated enteric bacteria including *E. coli* 41.0 % of municipal tap water samples and observed that 82.4 % of these samples did not comply with the WHO guidelines.

It has been frequently considered that *Salmonella* spp. are opportunistic and potential pathogenic bacteria of water bodies in warm climatic zones and poses a great risk for human and animal health (heinitz *et al.,* 2000). In present study, Prevalence of *Salmonella* spp. in the water samples of different site in Dhaka city is also alarming. About 37 samples out of 100 samples was positive for *salmonella* spp. The water sources considered for this study were also from the different cattle farm located at Adabor, Dhaka Uddan, Bosila, Hazaribagh, Mirpur, Keraniganj, Gabtoli in Dhaka city. The rate of isolation of *salmonella* spp. was the highest (50%) for Gabtoli followed by Bosila (46.67%), Hazaribagh (37%) and least for Dhaka Uddan (26.67%). *Salmonellae* has been detected in different percentages by several authors in surface waters, as varied as 8.5% (Jokinen *et al.* 2011); 15.4% (Adingra *et al.* 2012); 18.0% (Yam *et al*. 2000); 62.9% (Anselmo *et al.* 1999); 79.2% (Haley *et al.* 2009) and 96.0% (Rajabi *et al.*, 2011). This was perhaps because the presence and the abundance of *Salmonellae* in aquatic environments vary temporally (Haley *et al*., 2009) and is related to one or two a combination of sewage effluents, such as agricultural run-off and direct fecal contamination from natural fauna (Abulreesh, 2012). Additionally, the possibility of intermittent findings or of detecting different serovars in the same site of sampling suggests the heterogeneity of the aquatic environment (Rolland and Block, 1980). Climate is one factor that might explain the differences in the abundance and diversity of *Salmonella* spp. isolates between different locations (Gorski *et al.*, 2011). Higher prevalence was found from a study in Mexico. In that study, a total of 138 water samples from all sampling sites (A, B, C, D, E and F) were obtained to evaluate the presence of *Salmonella* spp., of which 111 (80.4%) were positive. Sampling sites A and E showed the highest prevalence of the bacteria, with 95.65% and 91.30%, respectively and site F had the lowest prevalence with 60.87% (Maribel Jimenez *et al*.,2014).

Lower prevalence of *Salmonella* spp. was also found from Bhatta *et al.* (2007), who reported that out of 300 samples only 14% were positive for *Salmonella* and the organisms were identified as *S. typhi, S. paratyphi* A, *S. typhimurium* and *S. enteritidis*. In Nepal, Shrestha *et al.* (2011), reported 4.7% occurrence of *Salmonella,* of which 1 (10%) was *S. paratyphi* A and 9(90%) were non-typhi, in 86 water samples collected from urban water supply system of Kathmandu and Rasheed *et al.* reported that 5% of the drinking water samples collected from Lahore, Sargodha and Sahiwal were contaminated with *Salmonella* which is lower than that of this study.

4.12 Results of antibiogram assay

A total of two isolates such as. *E. coli* and *Salmonella* spp. were subjected to antibiogram assay. The results of antibiogram assay are presented below.

Table 7: Resistivity profile of *E. coli* isolated from the cattle farm water of Dhaka city

Antimicrobial agents and Number of Sample Tested	Resistant (mm)	Intermediate (mm)	Sensitive (mm)	Interpretation
Streptomycin (10)				Resistant-26%
	≤15	16-21	≥22	Intermediate-2%
				Sensitive-72%
Ciprofloxacin (10)				Resistant-25%
	≤ 20	21-30	≥31	Intermediate-22%
				Sensitive-53%
Ampicillin (11)				Resistant-38%
	≤ 12	13-19	≥20	Intermediate-11%
				Sensitive-51%
Gentamycin (10)				Resistant-43%
	≤11	12-14	≥15	Intermediate-20%
				Sensitive-37%
Tetracycline (12)				Resistant-26%
	≤12	13-14	≥15	Intermediate-7%
				Sensitive-67%

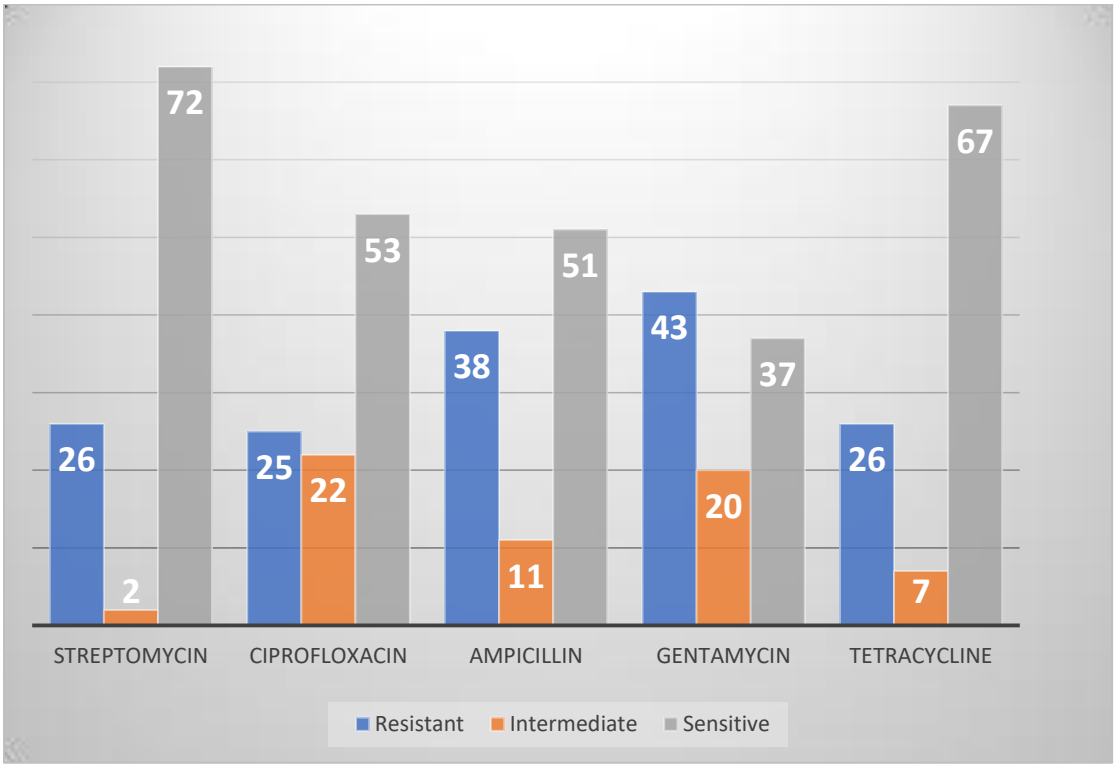

Figure 19: Graphical representation Resistivity profile of *E. coli* isolated from water samples.

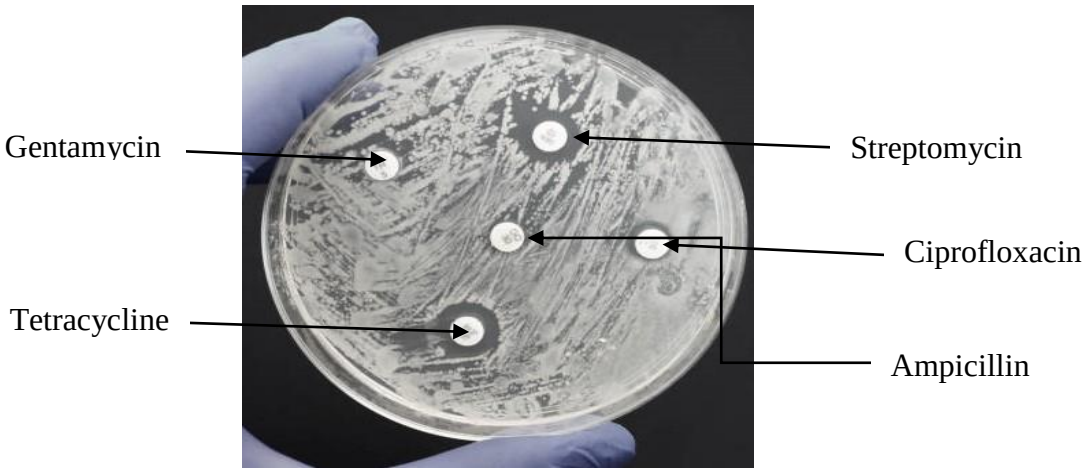

Figure 20: Antibiotic Sensitivity Test Methods in Detecting Antimicrobial Resistance using Petri dish.

Antimicrobial drug resistance/sensitivity tests are performed for those organisms that cause infections in humans or animals and those that may show resistance to commonly used antibiotics. The antibiotics used in this study for testing resistance of *E. coli* isolates were streptomycin (Str), ampicillin (Amp), ciprofloxacin (Cip), gentamycin (Gen) and tetracycline (Te). In the present study, of the 46 *E. coli* isolates,

all were resistant to at least one antibiotic. Among the 46 antibiotic resistant isolates, highest number of isolates showed resistance to gentamycin (43 %) followed by ampicillin (38%) and tetracycline (26 %). Least number of isolates was resistant to the ciprofloxacin (25 %) and streptomycin (26%). High resistance to tetracycline may be attributed to the fact that it is a product of naturally occurring compounds and bacteria can be exposed to it in nature and develop resistance even outside any host (Sayah *et al*., 2005). The observed prevalence was lower than that reported in previous studies of antibiotic resistant *E. coli* from water sources. Studies of Akharaiyi *et al.* (2007) showed high resistance to amoxicillin (75 %) in *E. coli* isolated from rainwater in Nigeria. Dhanji *et al*. (2011) reported extremely high resistance to ciprofloxacin (100 % of isolates) and to gentamycin (only in ESBL negative strains) among *E. coli* isolated from river Thames. Ramteke *et al*. (1990) observed the widespread occurrence of antibiotic resistance in surface water and ground water samples collected from springs, streams, dug wells, tube wells from rural areas of Uttar Pradesh, Himachal Pradesh, West Khasi hills and Meghalaya. They observed antibiotic resistance of *E. coli* was 90% of that's isolated from ground water and 100% of coliforms isolated from surface water. Pathak and Gopal (1994) reported antibiotic resistance in 63.6% of coliforms isolated from springs, streams and dug wells in Rajouri district of Jammu and Kashmir.

However, prevalence of resistance levels lower than those observed in this study has also been accounted in some investigations. Roe *et al.* (2003) could isolate only a small number of *E. coli* resistant to tetracycline (9 %) and gentamycin (0.3 %) from water used for irrigation purpose. Sayah *et al*. (2005), in an investigation of surface waters of Red Cedar, Michigan observed that none of the isolated *E. coli* (0.0 %) was resistant to gentamycin, tetracycline. Differences observed in the resistance towards individual antibiotics may be due to the varying degree of exposure of the hosts, which creates chances for the development of *E. coli* isolates that are antibiotic resistant or sensitive.

Table 8: Resistivity profile of *Salmonella* spp. isolated from the cattle farm water of Dhaka city

Antimicrobial agents	Resistant	Intermediate	Sensitive	Interpretation
Streptomycin (8)				Resistant-26%
	≤15	16-21	≤22	Intermediate-53%
				Sensitive-21%
Ciprofloxacin (10)				Resistant-14%
	≤20	21-30	≤31	Intermediate-42%
				Sensitive-44%
Ampicillin (12)				Resistant-31%
	≤12	13-19	≤20	Intermediate-13%
				Sensitive-56%
Gentamycin (10)				Resistant-31%
	≤11	12-14	≤15	Intermediate-10%
				Sensitive-59%
Tetracycline (13)				Resistant-27%
	≤12	13-14	≤15	Intermediate-21%
				Sensitive-52%

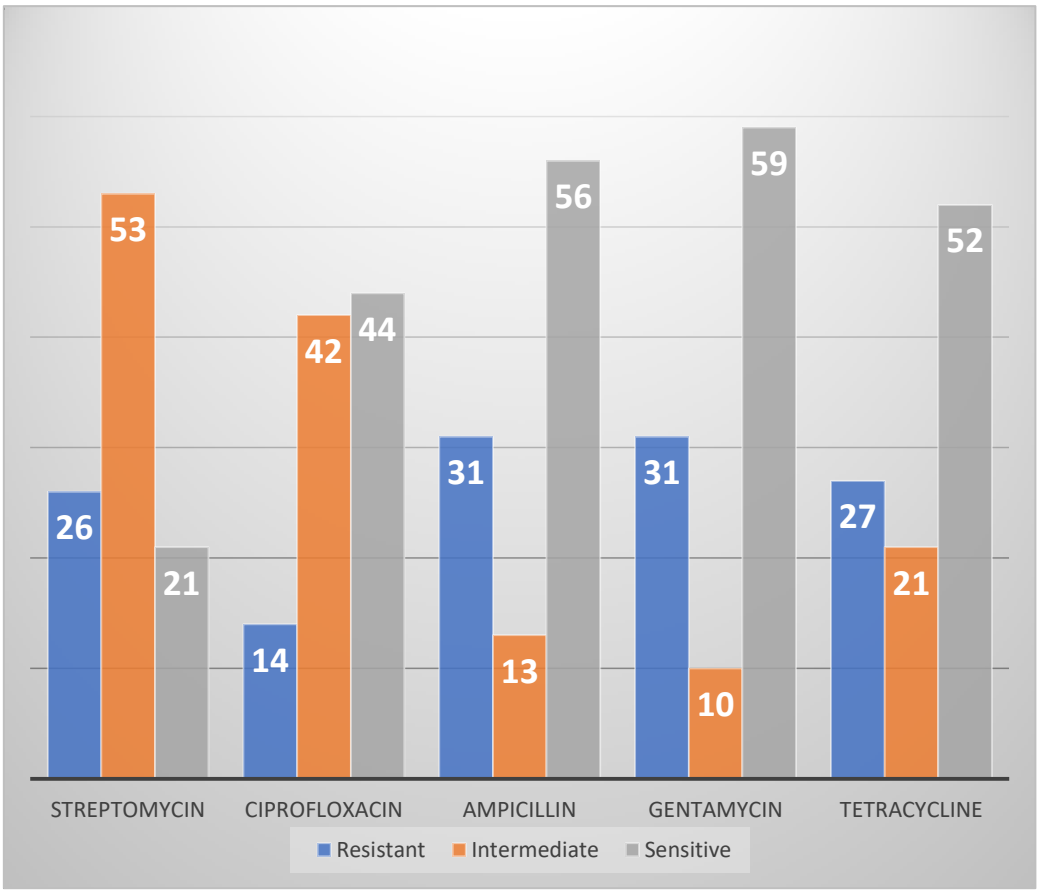

Figure 21: Graphical representation resistivity profile of *Salmonella* spp. isolated from water samples.

The present study, antibiogram of *salmonella* spp. has been observed. Five commercially available antibiotic were used. The used antibiotics were streptomycin, ampicillin, ciprofloxacin, gentamycin and tetracycline. All isolated *salmonella* spp. were resistant to at least one antibiotic. Among the 37 antibiotic resistant isolates highest number of isolates showed resistance to gentamycin (31 %) and ampicillin (31%) followed by tetracycline (27 %). Least number of isolates was resistant to the ciprofloxacin (14 %) and streptomycin (26%). Ahmed *et al.* reported that about 87.5% isolates were found resistant to ampicillin which is higher than present study. In Malaysia Gunasegaran *et al.* reported that all (100%) of the *Salmonella* isolates were resistant to ampicillin, tetracycline and chloramphenicol of a farm water. White *et al. (2003),* examined that most of the *Salmonella* isolates were susceptible to antimicrobials tested. Twenty-eight (36%) *Salmonella* isolates were resistant to at

least one antimicrobial and 10 (13%) isolates displayed resistance to four or more antimicrobials. Similar result to this study was also reported by Prasai *et al., (*2007). In that report resistance of *Salmonell*a to ciprofloxacin was 12 % followed by ampicillin 35 % and streptomycin 21 %. Slight differences occurred due to variation in sampling site, methods and materials applied.

CHAPTER 5

SUMMARY AND CONCLUSION

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The present study was conducted for isolation and identification of *E. coli* and *Salmonella* spp. microorganisms from cattle farm water samples and also to perform a comparative study to determine the sensitivity and resistance pattern of the isolates to different commercially available antibiotics. The study area was Adabor, Dhaka Uddan, Bosila, Hazaribagh, Mirpur, Keraniganj and Gabtoli which are located at Dhaka city.

After collection, the samples were subjected to various tests and experiments for isolation and identification of organism from cattle farm water. *E. coli* and *Salmonella* isolation was performed by propagating the organisms in nutrient broth and nutrient agar followed by culture on different agar media such as MacConkey agar, EMB agar and SS agar for the determination of their colony characteristics. A total of 100 water samples were collected from 7 different regions of Dhaka city. Total viable count (TVC) and TCC were done by ten-fold dilution method. 46 % samples were positive for *E. coli* and 37% samples were positive for *Salmonella* spp. and the rest samples couldn't be identified in this study. They were identified on the basis of colony morphology and Gram's staining technique. Biochemical properties of the isolates were studied by Catalase test, MR test, VP test and indole production test.

The study was also extended to investigate the sensitivity and resistance pattern of the *E. coli* & *Salmonella* spp. isolates to different antibiotics. This study revealed that there were considerable variations among the isolates of different sources in respect of antibiotics sensitivity and resistance pattern. High percentage of *E. coli* isolates were sensitive to streptomycin (72%), tetracycline (67 %) and ciprofloxacin (53%) while most of the *E. coli* isolates were resistant to Gentamycin (43%) and ampicillin (38%).

In case of *Salmonella spp.* isolates, good sensitivity was found against gentamycin (59%), ampicillin (56%) and ciprofloxacin (53%) while most of the *Salmonella* spp. isolates were resistant to ampicillin (31%) and tetracycline (27%).

It is assumed that one or more drug resistant clones have gradually acquired resistance to other drugs by conjugation with multi-drug resistant (MDR) strains.

From the present study it may be concluded that

(a) Water samples collected from different cattle farm in Dhaka city are infected with *E. coli* and *Salmonella* spp. Identified bacteria from the samples were *E. coli* and *Salmonella* spp.

(b) *E. coli* infections of different animals and birds and also of human being may be treated effectively with ciprofloxacin, tetracycline and streptomycin. Infection with *Salmonella* spp. can be treated with ciprofloxacin followed by streptomycin and gentamycin.

(c) further analysis may be done to identify degree of pathogenicity of bacteria and to take precautionary measure.

LIMITATION

We started our research work before the outbreak of Covid-19 throughout the world. Due to Covid-19 outbreak in 2020, our research work became so tough to continue from when it declared as a global pandemic. Strict lockdown was declared throughout the country. Our university halls were permanently closed by the university administration. In that crucial situation we couldn't continue our study. The entry of lab wasn't available, it was a crucial problem to keep the continuation of our work. We took only five different antibiotics because of imposition of strict lockdown throughout the country. Shops were closed for long time. Due to prohibition of international trade and business throughout the world, antibiotics disk was not available in the market. After all, we didn't have enough facilities in our lab due to lack of instruments and sufficient funds.

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APPENDICES

APPENDIX I

COMPOSITION OF DIFFERENT MEDIA

1. Nutrient broth (NB)

Peptic digest of animal tissue ……5.00gm
Sodium chloride…… 5.00gm
Beef extract …… 1.50gm
Yeast extract …… 1.50gm
Distilled water…… 1000 ml
Final pH (at 25ºC) …… 7.4+0.2

2. Eosin Methylene Blue (EMB) agar

Peptone…… 10.00gm
Dipotassium Phosphate…… 2.00gm
Lactose …… 5.00gm
Sucrose …… 5.00 gm
Agar ……. 13.50 gm
Eosin ……. 0.40 gm
Methylene blue …… 0.065 gm
Distilled water……1000 ml
Final pH (at 25ºC) ……7.4+0.2

3. MacConkey (MC) agar

Lactose ……10.00gm
Peptone ……20.00gm
Sodium chloride…… 5.00gm
Bile salts ……5.00gm
Neutral red …… 0.075 gm
Agar …… 12.00gm
Distilled water…… 1000 m

4. Mannitol salt agar

Lab-Lemco Powder……1.00gm

peptone …… …….. 10.00 gm

Mannitol …… 10.00 gm

Phenol red …… 0.025 gm

Agar …… 15.0 gm

Distilled water …… 1000 ml

5. Salmonella-Shigella (SS) agar

Peptic digest of animal tissue ……5.00 gm

Beef extract …….. 5.00 gm

Lactose ……. 10.00 gm

Bile salts mixture ……. 8.50 gm

Sodium citrate ……. 10.00 gm

Sodium thiosulfate …… .8.50 gm

Ferric citrate …… 1.00 gm

Brilliant green …… 0.00033 gm

Neutral red …… 0.025 gm

Agar …….. 15.00 gm

Distilled water…… 1000 ml

Final pH (at 25ºC) …… 7.0 ± 0.2

6. Nutrient agar (NA)

Beef extract …….. 3.00 gm

Peptone …….. 5.00 gm

Sodium chloride……5.00 gm

Agar …… 20.00 gm

Distilled water……1000 ml

Final pH (at 25ºC) ……7.1 ± 0.1

7. MR-VP medium (Himedia, India)

Buffered peptone……7.00 gm

Dextrose …… 5.00 gm

Dipotassium phosphate…… 5.00

APPENDIX II

COMPOSITION OF DIFFERERNT REAGENTS

1. Peptone water

Peptone …… 1.00 gm

Distilled water…… 1000 ml

2. Kovac's reagent for indole preparation

Pdimethyl amino benzaldehyd……5.00gm

Amyl alcohol …….. 75.00 ml

Conc. HCl …….. 25.00 ml

3. V-P reagent-1

5% alpha-napthol in absolute ethyl alcohol

4. V-P reagent-2

Creatine ……. 0.30%

Cotton blue ……. 0.05g

5. Phosphate buffered saline

Sodium chloride…….. 8.00gm

Disodium hydrogen phosphate…… 2.80gm

Potassium chloride …… 0.20gm

Potassium hydrogen phosphate…… 0.20gm

6. Methyl red solution 1000 ml

Methyl red …… 0.05 gm

Ethanol (absolute) ……28.00 ml

Distilled water…… 22.00 ml

7. Phenol red

Phenol red dye…… 0.20gm

Distilled water…… 100 ml

Total Viable Count and total coliform count from the isolated sample

Serial no	Name of the Sample	Total Viable Count (TVC)(CFU/ml)	Total Coliform Count (TCC)(CFU/ml)
1	S1	3.8×10^4	3.1×10^4
2	S2	2.6×10^4	Nil
3	S3	6.5×10^3	4.9×10^3
4	S4	1.2×10^4	Nil
5	S5	5.6×10^4	7.5×10^3
6	S6	4.1×10^5	Nil
7	S7	3.5×10^4	Nil
8	S8	4.3×10^4	1.2×10^4
9	S9	1.2×10^5	Nil
10	S10	3.9×10^4	6.5×10^3
11	S11	3.40×10^4	Nil
12	S12	3.80×10^4	Nil
13	S13	5.3×10^3	Nil
14	S14	8.5×10^4	4.3×10^4
15	S15	6.7×10^3	1.08×10^3
16	S16	4.5×10	Nil
17	S17	4.5×10^3	2.8×10^3
18	S18	1.08×10^4	Nil
19	S19	4.8×10^4	Nil
20	S20	5.2×10^3	Nil
21	S21	3.5×10^4	1.9×10^4
22	S22	1.2×10^4	1.2×10^4
23	S23	6.1×10^3	Nil
24	S24	5.9×10^4	1.2×10^4
25	S25	3.9×10^5	5.8×10^4
26	S26	2.7×10^5	Nil
27	S27	4.2×10^4	Nil

28	S28	3.1×10^{5}	5.2×10^{4}
29	S29	5.5×10^{4}	Nil
30	S30	9.7×10^{4}	2.2×10^{4}
31	S31	2.08×10^{4}	Nil
32	S32	4.8×10^{4}	Nil
33	S33	6.6×10^{3}	Nil
34	S34	5.3×10^{5}	3.2×10^{4}
35	S35	4.8×10^{5}	Nil
36	S36	8.2×10^{4}	4.2×10^{4}
37	S37	8.9×10^{4}	Nil
38	S38	5.6×10^{5}	2.7×10^{4}
39	S39	5.1×10^{4}	Nil
40	S40	6.1×10^{4}	Nil
41	S41	1.5×10^{6}	Nil
42	S42	3.8×10^{4}	Nil
43	S43	5.9×10^{3}	2.1×10^{3}
44	S44	6.1×10^{4}	Nil
45	S45	9.9×10^{4}	6.8×10^{3}
46	S46	3.9×10^{5}	Nil
47	S47	6.1×10^{4}	5.9×10^{3}
48	S48	3.9×10^{5}	Nil
49	S49	5.3×10^{3}	Nil
50	S50	3.9×10^{4}	Nil
51	S51	7.3×10^{4}	5.7×10^{3}
52	S52	4.1×10^{3}	Nil
53	S53	3.9×10^{5}	9.3×10^{3}
54	S54	4.9×10^{4}	Nil
55	S55	5.3×10^{4}	2.5×10^{3}
56	S56	4.9×10^{4}	Nil

57	S57	7.2×10^{3}	3.3×10^{3}
58	S58	3.9×10^{4}	Nil
59	S59	3.7×10^{4}	4.3×10^{3}
60	S60	1.09×10^{4}	Nil
61	S61	4.2×10^{4}	Nil
62	S62	2.9×10^{5}	Nil
63	S63	6.2×10^{4}	Nil
64	S64	7.3×10^{3}	1.5×10^{3}
65	S65	2.6×10^{5}	9.5×10^{3}
66	S66	4.9×10^{4}	Nil
67	S67	8.4×10^{4}	Nil
68	S68	4.2×10^{5}	Nil
69	S69	4.5×10^{4}	1.9×10^{4}
70	S70	5.2×10^{3}	Nil
71	S71	9.1×10^{4}	3.8×10^{3}
72	S72	6.2×10^{4}	Nil
73	S73	4.9×10^{5}	Nil
74	S74	2.0×10^{5}	4.9×10^{4}
75	S75	4.8×10^{4}	Nil
76	S76	3.9×10^{3}	Nil
77	S77	5.7×10^{4}	5.8×10^{3}
78	S78	5.9×10^{4}	Nil
79	S79	2.9×10^{5}	Nil
80	S80	3.2×10^{5}	7.8×10^{4}
81	S81	4.9×10^{4}	Nil
82	S82	2.4×10^{5}	Nil
83	S83	4.9×10^{4}	2.6×10^{4}
84	S84	2.9×10^{5}	Nil
85	S85	6.2×10^{4}	Nil

86	S86	5.3×10^{3}	1.8×10^{3}
87	S87	3.4×10^{4}	Nil
88	S88	2.2×10^{4}	Nil
89	S89	4.4×10^{4}	Nil
90	S90	9.1×10^{3}	3.8×10^{3}
91	S91	4.2×10^{4}	Nil
92	S92	3.7×10^{5}	7.8×10^{3}
93	S93	3.2×10^{4}	Nil
94	S94	9.8×10^{4}	Nil
95	S95	6.3×10^{4}	4.9×10^{4}
96	S96	5.2×10^{5}	Nil
97	S97	6.1×10^{3}	Nil
98	S98	3.3×10^{5}	Nil
99	S99	6.2×10^{4}	Nil
100	S100	5.1×10^{4}	Nil